

Supplementary Information

Theragnosis by MiR-141-3p Molecular Beacon: Simultaneous Detection and Sensitization of 5-Fluorouracil Resistant Colorectal Cancer Cells through the Activation of TRIM13-Associated Apoptotic Pathway

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This PDF file includes:

1. Experimental section
2. Supplementary figures S1 to S5
3. Supplementary tables S1 and S2
4. Reference

1. Experimental Section

1.1 Cell culture

Human embryonic kidney 293 cells (HEK293) and human colon cancer cell lines including DLD1, and SNU175 (N-CCLs) were purchased from the Korean Cell Line Bank (KCLB, Chongno, Seoul, Korea). The cell lines were cultured on growth media that consisted of Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA), RPMI1640 with 10 % fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1 % antibiotics (Gibco) in a 5 % CO₂-humidified incubator. The DLD1 and SNU175 cell lines were harvested in the exponential growth phase, resuspended at a density of 10^6 cells mL⁻¹, and then treated with various dilutions of (5×10^6 , 10^6 , 5×10^5 , 10^5 , 5×10^4 , 10^4 , 5×10^3 , 10^3 , 5×10^2 , and 10^2) per well of 10 μ M 5-FU and 5 ng mL⁻¹ TGF- β by the limited dilution method, as described previously.¹ All cells were cultured in RPMI-1640 medium containing 10 % fetal bovine serum (FBS) in a humidified incubator at 37 °C and 5 % CO₂. The resulting chemoresistant colon cancer cell lines, named DLD1-C10 and SNU175-B12 (R-CCLs), were able to grow in the presence of 75 and 20 μ M 5-FU in RPMI-1640 medium containing 10 % fetal bovine serum (FBS) and 1 % antibiotics. Transfection of the oligonucleotides, miRNA MB, or exogenous miRNA was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions.

1.2 Design of MB

MBs for miR-141-3p and piR-126541 (a negative control) were designed to form a partially double-stranded oligonucleotide. The fluorophore-labeled reporter oligos of MBs containing its binding sequence complementary to target molecules were as follows: miR-141-3p MB, 5'-Cy3.0-

CCGCGACCATCTTTACCAGACAGTGTTA-3'; piR-126541 MB, 5'-Cy5.5-CCGCGAATCTATCACTCCTGAACTGAGATCAC-3'. The sequences of the quencher oligo were as follows: miR-141-3p MB, 5'-AGATGGTCGCGG-BHQ1-3'; and piR-126541 MB, 5'-ATAGATTTCGCGG-BHQ2-3'. Reporter and quencher oligos were purchased from Bioneer (Bioneer, Daejeon, Republic of Korea). 30 pM of each reporter oligo was annealed with 60 pM of its corresponding quencher oligo (molar ratio of 1:2) to form miR-141-3p MB and piR-126541 MB. For intracellular delivery of MB, 1×10^5 cells were transfected with MB using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

1.3 Characterization of MB

To evaluate the quenching efficiency of miR-141-3p MB or piR-126541 MB, 30 pM of each long oligo was reacted with different concentrations (0, 15, 30, 60 and 100 pM) of its corresponding short oligo, and incubated in a black tube for 1 h at RT. To determine the specificity of miRNA MB, 30 pM of miR-141-3p MB or piR-126541 MB was incubated with different concentrations (0, 10, 20, 50, 100 and 200 pM) of its corresponding exogenous miRNA, or 200 pM of a negative piRNA, in a black tube for 1 h at RT. The Cy3.0 fluorescence signal of each reporter oligo was measured at an excitation of 546 nm and emission of 563 nm, using a Varioskan Flash Fluorescent Microplate Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). To evaluate its stability, 100 pM of piR-36026 MB was incubated in phosphate-buffered saline (PBS) and DMEM culture media (Hyclone) at 37 °C for 4 days, then 200 pM of exogenous miR-141-3p was added to miR-141-3p MB-incubated RPMI1640 medium for an additional 1 h at 37 °C, whereupon the Cy3.0 fluorescence intensity was measured.

1.4 Confocal microscopic imaging

From each cell line, 5×10^4 cells were seeded onto 24-well plates containing 25-mm-diameter coverslips, and grown for 24 h at 37 °C. For the detection of miR-141-3p in the cell, various concentration of miR-141-3p MB (50 pM for HEK293 cells, and 0, 25, 50, 75, 100 and 200 pM for each CCL), or 200 pM of piR-126541 MB was transfected with Lipofectamine 2000, and incubated in a 5 % CO₂-humidified incubator for 6 h. Simultaneously, incremental concentration (0, 0.05, 0.1, 0.5 and 1 nM) of exogenous miR-141-3p was co-transfected to each well plate. Exogenous piR-126541 (1 nM) was used as a negative control. After washing with PBS twice for 10 min, the cells were fixed with 500 μ L of 3.7 % formaldehyde (Sigma Aldrich). Then, they were washed three times with PBS for 10 min, and capped with coverslips, using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA, USA). Quantitative fluorescence intensities and confocal microscopy images for miR-141-3p MB (excitation/emission: 546/563 nm), and DAPI (excitation/emission, 305/460 nm) were measured using a Varioskan Flash Fluorescent Microplate Fluorometer and LSM700 confocal laser scanning microscopy (Carl Zeiss, Weimer, Germany), respectively. Quantitative data are presented as the mean $\pm$ SD calculated from triplicate samples and significant differences between samples were assessed using a Student's t-test.

1.5 Quantitative reverse polymerase chain reaction (qRT-PCR) analysis

Small RNA was isolated from cell lines using the mirVanaTM isolation kit (Ambion, Grand Island, NY, USA). For qRT-PCR of small RNA, RNA samples were reverse transcribed using the NCodeTM VILOTM miRNA cDNA Synthesis Kit (Invitrogen) for poly(a) tailing and cDNA synthesis. For qRT-PCR of piRNA target genes, total RNA was isolated using TRIzol (Invitrogen). Reverse

transcription was carried out using a high capacity RNA to cDNA kit (Applied Biosystems, Grand Island, NY, USA). qRT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Odessa, TX, USA) and StepOnePlus™ Real-Time PCR System (Applied Biosystems). The relative amounts of each mature miRNA were normalized versus 5Sr RNA, and target genes were normalized versus GAPDH. Primer sequences of mRNA and miRNA are listed in Tables S1 and S2 of the SI.

1.6 Immunoblotting analysis

Cell lines were collected and lysed with RIPA Lysis Buffer (Thermo Fisher Scientific Ind., Odessa, TX, USA). Protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Ind., Waltham, MA, USA). Equivalent amounts (30 µg) of protein from each lysate were separated using SDS-PAGE, and transferred to PVDF (Milipore, Billerica, MA, USA) for Western blotting. The membranes were washed 3 times with Tris-buffered saline (TBS, ELPIS, Daejeon, Korea) containing 0.1 % Tween 20 (Sigma Aldrich) (TBST). After blocking with TBST containing 5 % skim milk (Wako, Osaka, Japan) for 1 h, the membranes were incubated with the appropriate primary antibody in TBST containing 5 % skim milk at 4 °C overnight. All primary antibodies were diluted in an appropriate concentration of 5 % skim milk containing TBST. After treatment with CDKN1A, PTGS2, TRIM13, cleaved-Caspase 3, p-ERK, p-AKT, and β-actin (all from Cell Signaling Technology, Danvers, MA, USA) or IL8 and PLAU (both from Abcam, Cambridge, UK), transfer membranes were washed 3 times with TBST for 30 min, followed by goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (diluted 1:4,000, Sigma Aldrich) for 2 h at RT, and washed 3 times with TBST for 30 min. The membranes were developed using the ECL Buffer (Thermo Fisher Scientific Ind.).

1.7 Cell viability assays

Each CCL and HEK293 was seeded at 3×10^3 cells per well in 96-well white flat-bottomed plates. After 24 h of incubation, the cells were treated with incremental concentrations of 5-FU (0, 1, 10, 100, and 1,000 μM) and miR-141-3p MB (0, 25, 50, 100, and 200 pM) in 10 % FBS-supplemented RPMI-1640 for 72 h. These cells were exposed to 0.5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich), and incubated for 2 h at 37 °C, and then 100 μL of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to solubilize the formazan crystals. The optical density (OD) was read at 570 nm, using a Varioskan Flash microplate reader (Thermo Fisher Scientific Ind.). Data were analyzed with SigmaPlot software (SYSTAT, Chicago, IL, USA), using Logistic 3 parameter analysis to determine IC_{50} of the chemotherapeutic agents.

1.8 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) was used to measure the molecular dynamics of miR-141-3p MB within the cytoplasm. For FRAP experiments, 1×10^5 of DLD1 and DLD1-C10 cells were plated on glass tissue culture dishes (SPL, Gyeonggi-do, Korea) and allowed to adhere to plates for 24 h. The cells were transfected with 200 pM of miR-141-3p MB using Lipofectamine 2000. FRAP studies were performed in 6 h after transfection on a confocal laser scanning microscope. The images were acquired in photobleaching mode (Confocal Software ZEN 1012; Carl Zeiss); $\times 400$ images were acquired at excitation 546 nm to 100 % in order to bleach a 2 μm diameter circular region of interest. Before bleaching, three pre-bleach scans were acquired to establish baseline fluorescence intensity, followed by 1 sec of bleaching. The percent recovery was calculated with the formula: (time dependent fluorescence value / fluorescence value of pre-bleach) $\times 100$ = recovery rate. Recovery was monitored until a recovery plateau is achieved. Raw

intensity values were exported into spreadsheet software (Excel; Microsoft, Redmond, WA, USA), background subtracted, and corrected for photobleaching effects during scanning. Three separate FRAP experiments were performed.

1.9 Gene expression profiling

Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, USA), purified using RNeasy columns (Qiagen, Valencia, USA) according to the manufacturers' protocol. After processing with DNase digestion, clean-up procedures, RNA samples were quantified, aliquot and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA). 750 ng of labeled cRNA samples were hybridized to each Human HT12 expression v.4 bead array for 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the

manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0)). Probes signal value was transformed by logarithm and normalized by quantile method.

1.10 Bioinformatic analysis

The microarray gene expression data of R-CCLs were compared with N-CCLs, and the genes showing at least 2.0-fold change were regarded as differentially expressed. The list of tumor suppressor genes associated with colon cancer was obtained from TSGene website (<https://bioinfo.uth.edu/TSGene/>). MiRNAs putatively having sequence for binding with *TRIM13* were predicted by the MicroRNA web resource (www.microrna.org). The putative miRNA binding site on *TRIM13* was further investigated by miRanda version 3.3a (Memorial Sloan-Kettering Cancer Center, New York, NY, USA), applying a stringent alignment score (≥ 100) and energy threshold (≤ -20.0 kcal M⁻¹).

1.11 Reporter gene assay

To visualize the regulation of *TRIM13* expression by miR-141-3p and its MB, pMIR-GFP system (Cell Biolabs, San Diego, CA, USA) containing an open reading frame of green fluorescence protein (GFP, excitation/emission, 475/509 nm), regulated by cytomegalovirus (CMV) promoter, was used. The sense and antisense primers for sequences of miR-141-3p binding region of *TRIM13* and its mutant form as a control, which has 8 base pair deletion at the 3'-end to inhibit binding with miR-141-3p, were annealed, and inserted between the unique *Bam*HI and *Eco*RI sites right after the stop codon of the GFP, to construct reporter vectors sensing *TRIM13* mRNA (CMV/GFP-*TRIM13*) and *TRIM13* mutant (CMV/GFP-*TRIM13*mt). Annealing was performed with a mixture of 20 pM of oligonucleotides and 60 μ L of annealing buffer containing 1 $\times$ Tris-ethylenediamine

tetraacetic acid buffer and 50mM sodium chloride, under the following conditions: 95 °C for 4 min and 70 °C for 10 s, followed by slow cooling in a 70 °C water bath for 90 min. The constructs were transfected into miR-141-3p MB-treated cells using Lipofectamine 2000. Transfection was conducted using 1 mg of constructs and 1 µL of Lipofectamine 2000 per well. The null vector without *TRIM13*-binding sequence (CMV/GFP) was used as control. All transfections were performed in triplicate. Quantitative fluorescence intensities of GFP were measured by confocal microscopy imaging.

1.12 Cell wound and healing assay

1×10^5 DLD1 or DLD1-C10 cells per well were transfected with miR-141-30 MB, and treated with 5, 10, and 20 µM of 5-FU in 24-well plates. Cells were scratched using a cell scraper (SPL, Pocheon, Korea), to generate ~1,200 µm-width wounds. The plates were then placed into a Tsview 7 (Tucsen, Fuzhou, China) for 72 h, to automatically capture the wound images at the exact same location. The data were analyzed by wound width or relative wound width. SNU175 and SNU175-B12 cells were excluded from wound healing assay due to its suspension growth pattern which is not suitable for experiment.

2. Supplementary figures

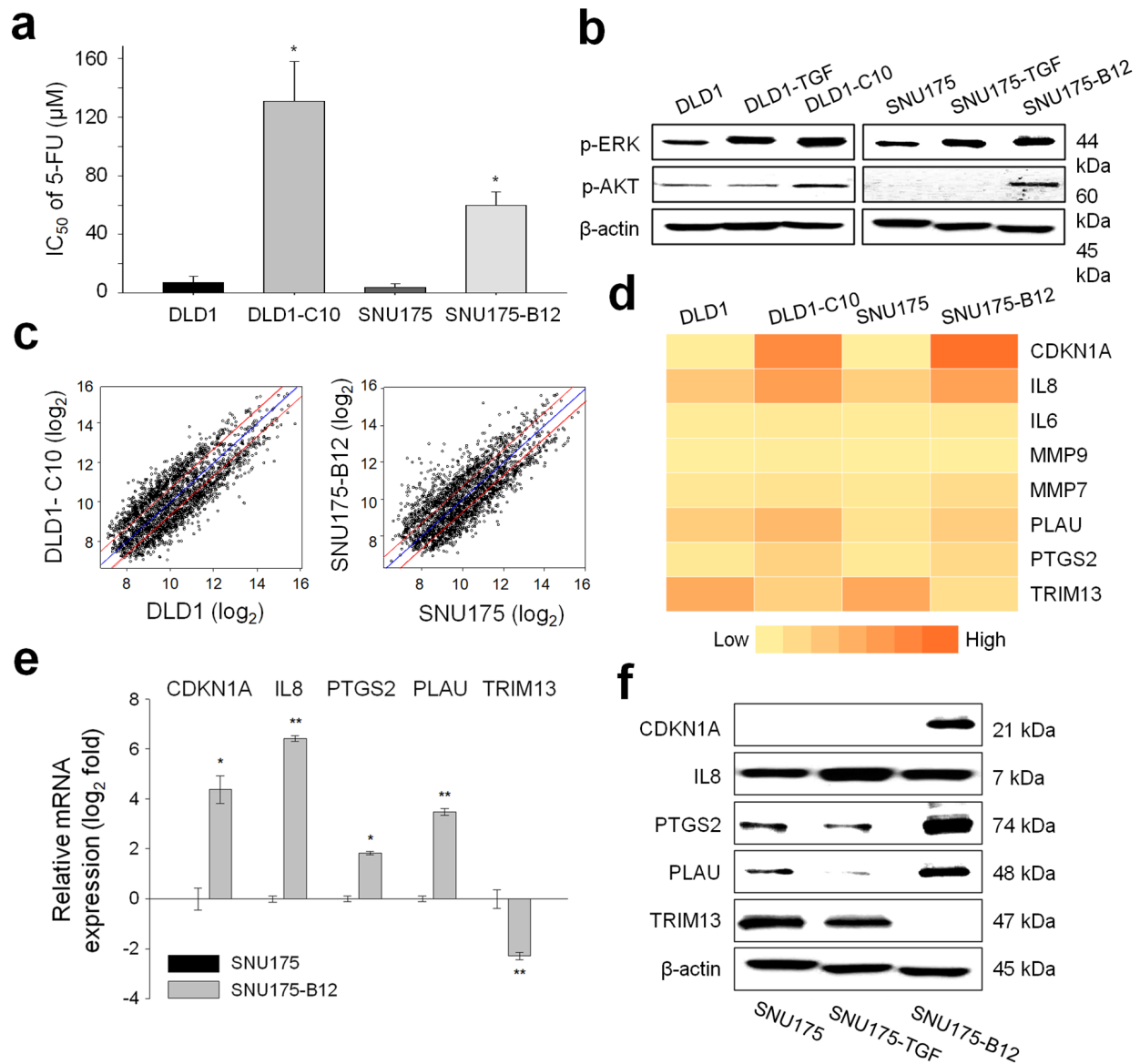


Fig. S1 (a) The half maximal inhibitory concentration (IC₅₀) of 5-FU for R-CCLs were significantly increased (MTT assay) (* $p < 0.01$). (b) The expression levels of p-ERK and p-AKT, representing the cell viability, were higher in R-CCLs than N-CCLs or the cells treated with TGF- β alone (immunoblotting). (c) The scatter plot showing differential gene expression between R-CCLs and N-CCLs. The spots between the red lines indicate fold-change values of the genes

between 0.5- and 2-fold. (d) TGF- β /SMAD pathway genes, except *IL6* and *MMP9*, were up-regulated, and *TRIM13* was the most down-regulated tumor suppressor gene in R-CCLs (microarray analysis). (e) qRT-PCR, and (f) immunoblotting revealed significantly increased levels of mRNAs and proteins in TGF- β /SMAD pathway associated with chemoresistance and decreased TRIM13 expression in SNU175-B12 (* $p < 0.05$, ** $p < 0.01$).

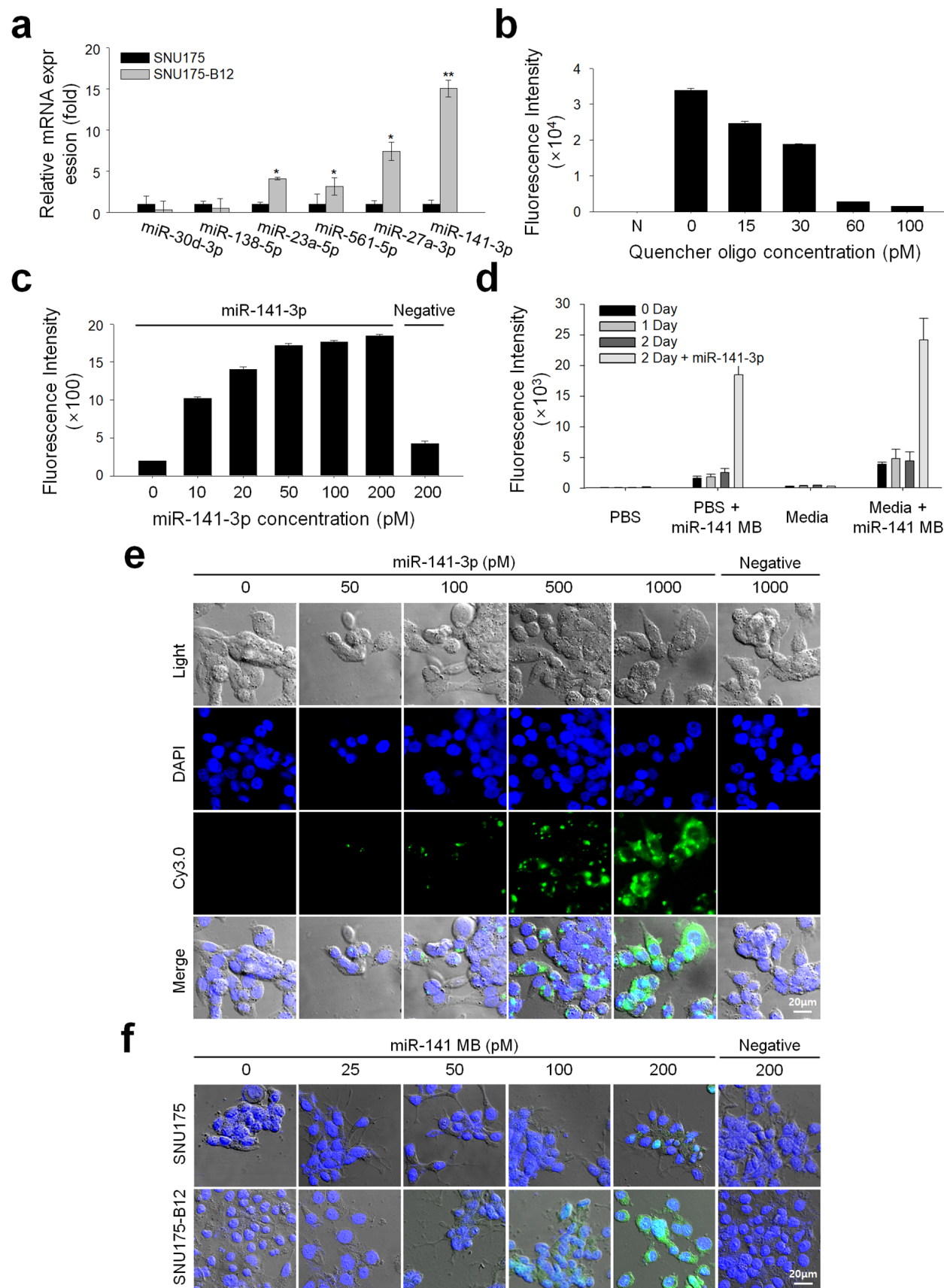


Fig. S2 (a) Among the 6 candidate miRNAs, miR-141-3p was most increased in SNU175-B12 (qRT-PCR; $*p < 0.05$, $**p < 0.01$). (b) Incremental concentrations of the quencher oligonucleotide were conjugated to 30 pM of reporter oligonucleotide. A dose dependent quenching effect of the Cy3.0 fluorescence intensity was observed in the microtube. (c) Various concentrations of exogenous miR-141-3p and, as a negative control, 200 pM of exogenous piR-126541 were incubated with 30 pM of miR-141-3p MB in the microtube. As concentration of the miR-141-3p increases, the fluorescence signal of miR-141-3p MB increased. (d) To test the stability of miR-141-3p MB, 100 pM of miR-141-3p MB was incubated in PBS and culture media (DMEM) for 0 to 2 days, and fluorescence intensity was measured. There was no significant change of fluorescence signal without miR-141-3p for 2 days, which suggests that miR-141-3p MB is so stable that spontaneous separation of quencher oligonucleotide hardly occurs without miR-141-3p for 2 days in the tube. (e) miR-141 MB sensitively and specifically detected miR-141-3p, exogenously transfected into HEK293. (f) miR-141 MB dose-dependently detected endogenous miR-141-3p in SNU175-B12 (confocal microscopic images; scale bar, 20 μ m).

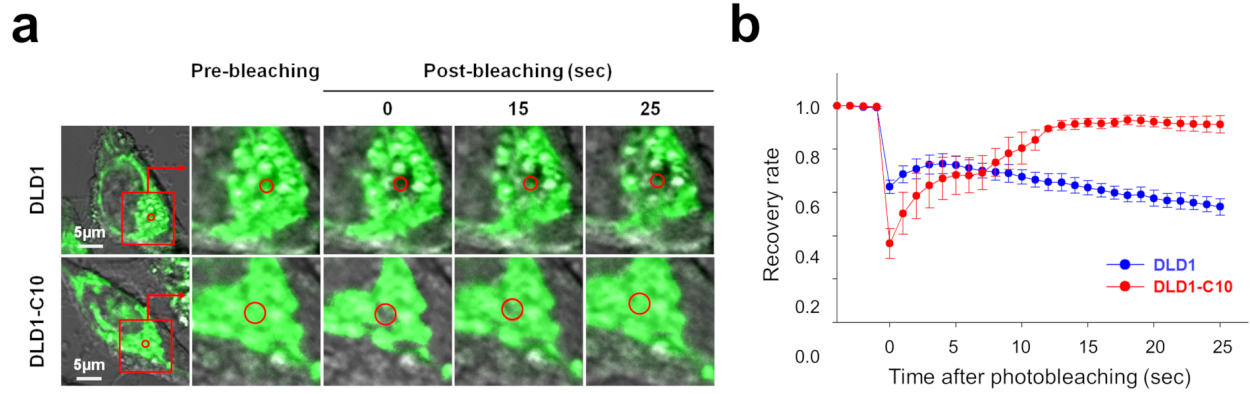


Fig. S3 (a) DLD1 and DLD1-C10 cells were transfected with 200 pM of miR-141-3p MB, then the red circle area of each cells was bleached for 1 s. Time series confocal images demonstrated almost complete recovery of fluorescence in DLD1-C10 cell, in contrast with DLD1 cell in which fluorescence signal faded out (scale bar, 5 μ m). (b) The fluorescence intensity of red circle area in DLD-C10 cell recovered 91.9 % of pre-bleaching level in 25 s, while maximally 73.3 % in DLD1 cell which was decreased with time.

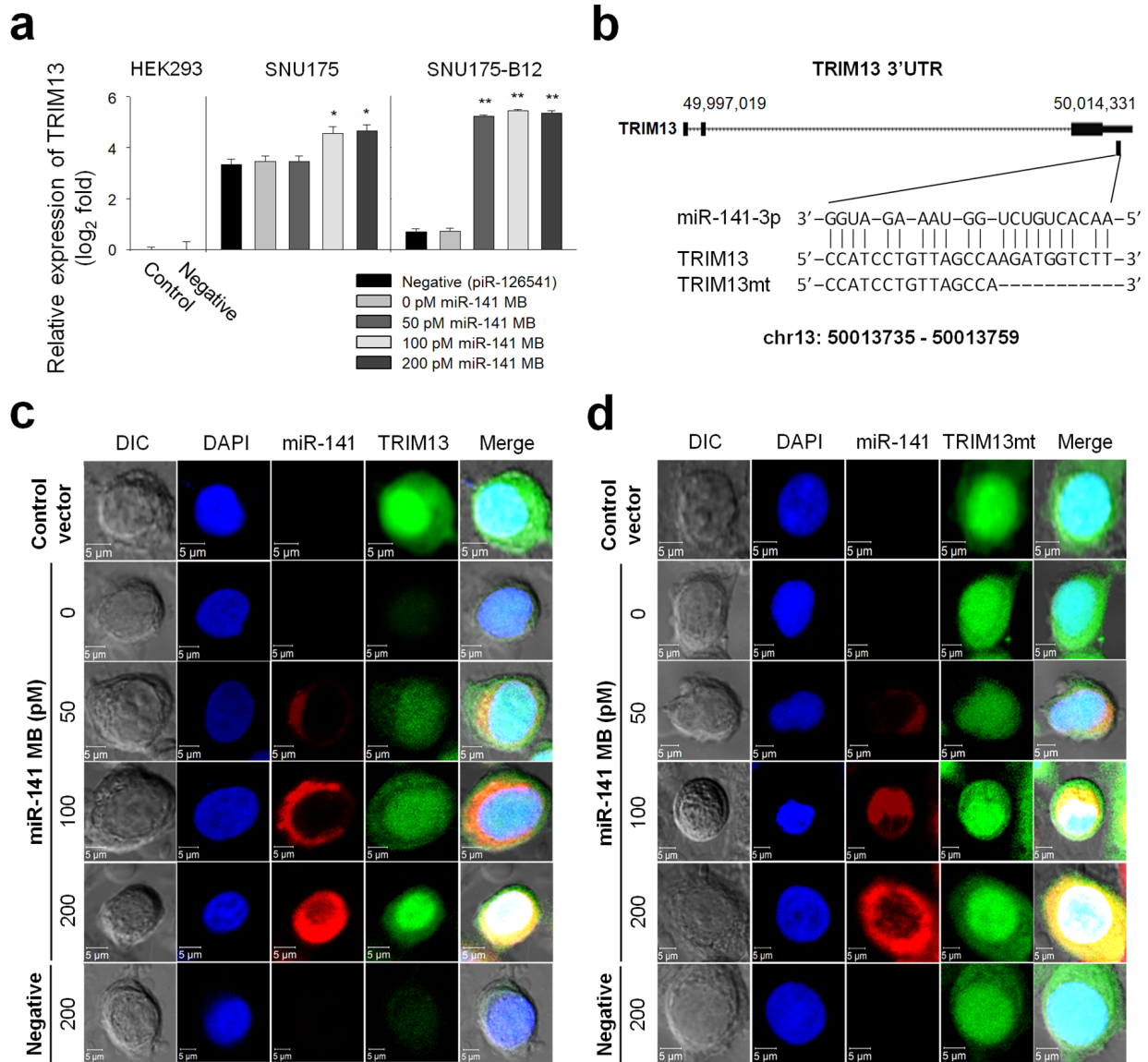


Fig. S4 (a) Transfection of incremental concentration of miR-141-3p MB (0, 50, 100, and 200 pM) resulted in proportional increase of *TRIM13* mRNA expression, more steeply in SNU175-B12 compared to SNU175 (qRT-PCR; * $p < 0.05$, ** $p < 0.01$). (b) For reporter gene analysis, the sequence of miR-141-3p binding region (*TRIM13*) was predicted using miRanda algorithm and its mutant form (*TRIM13mt*) was designed to be unable to bind miR-141-3p by 8 base pair deletion at the 3'-end. (c, d) The results of reporter gene analysis using SNU175-B12 were similar to those

of DLD1-C10 (confocal microscopic images; scale bars, 5 μ m).

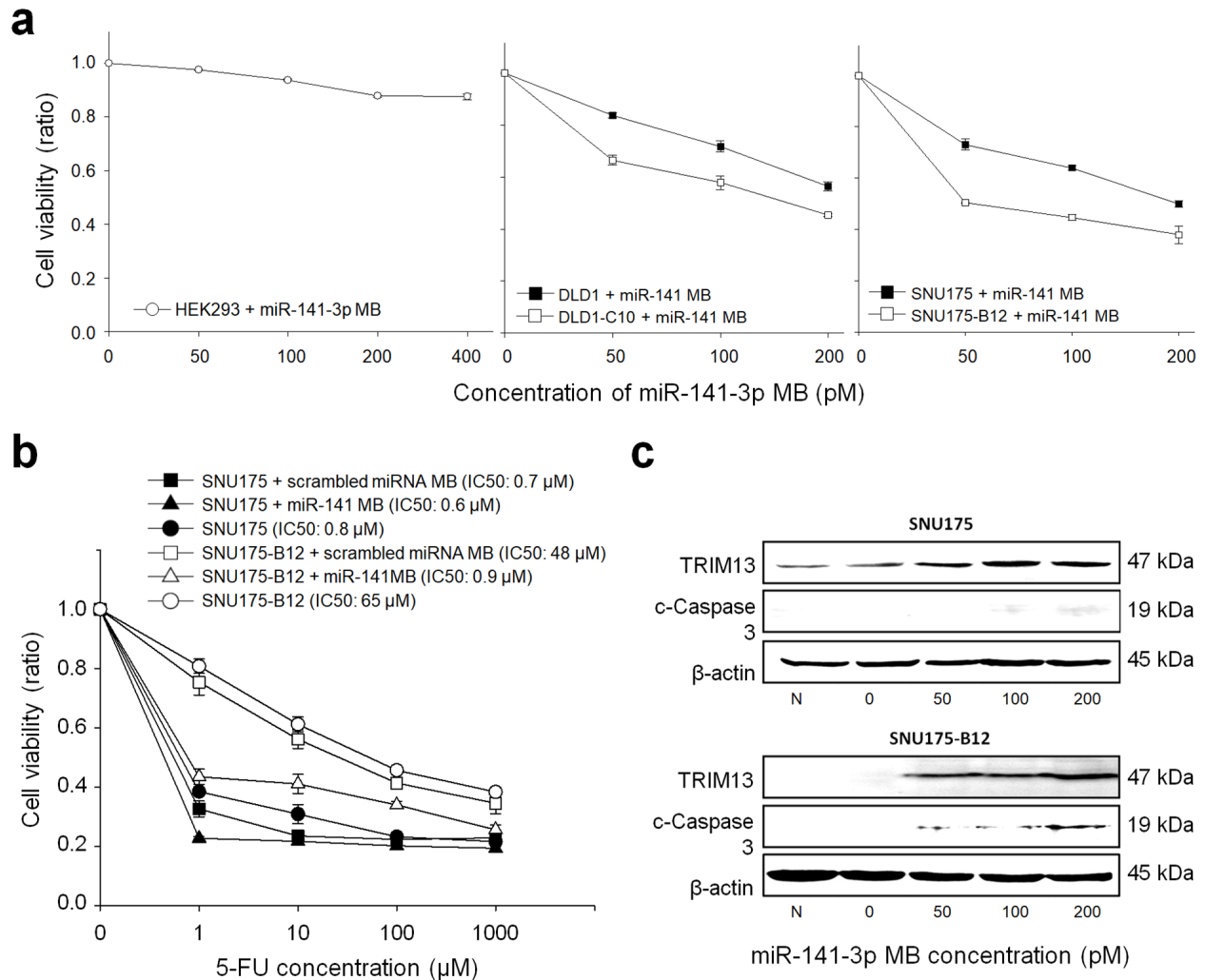


Fig. S5 (a) When treated with incremental concentrations (0, 50, 100, and 200 pM) of miR-141 MB alone, the viabilities of R-CCLs were reduced more steeply than that of N-CCLs in dose-dependent manner, while no significant change was observed in the viability of HEK293 cells (MTT assay). (b) Transfection of 100 pM miR-141 MB significantly lowered IC₅₀ of 5-FU on the SNU175-B12 cells from 65 μ M to 0.9 μ M, while on the SNU175 cells, enhancement of cytotoxic effect of 5-FU (IC₅₀) by transfection of miR-141 MB was not significant (from 0.8 μ M to 0.6 μ M) (MTT assay). (c) Immunoblotting demonstrated that transfection of miR-141 MB

dose-dependently restored TRIM13 protein expression in SNU175-B12 accompanied by activation of apoptotic pathway represented by cleaved caspase-3 (c-Caspase 3) expression, while changes of TRIM13 or c-Caspase 3 in SNU175 were not significant.

3. Supplementary Tables

Table S1. Primer information for qRT-PCR analysis for mRNAs

Gene symbol	Primer sequence (5' to 3')	
	Forward	Reverse
<i>CDKN1A</i>	GGAAGACCATGTGGACCTGT	GGATTAGGGCTTCCTCTTGG
<i>IL8</i>	GCAGAGGCCACCTGGATTGTGC	TGGCATGTTGCAGGCTCCTCAGAA
<i>PTGS2</i>	AGCTTTCACCAACGGGCTGGG	AAGACCTCCTGCCCCACAGCAA
<i>PLAU</i>	GCCCTGGTTTGCGGCCATCT	CGCACACCTGCCCTCCTTGG
<i>TRIM13</i>	TGCAAGCATATGACCCAGAGATC	GTTCTTCATTAAAGGGCTTGCAGG
<i>GAPDH</i>	GCCTCAAGATCATCAGCAATGCCT	TGTGGTCATGAGTCCTTCCACGAT

Table S2. Primer information for qRT-PCR analysis for miRNAs

miRNA symbol	Primer sequence (5' to 3')
RT6- miR-30d-3p	TGTCAGGCAACCGTATTCACCGTGAGTGGTGCAGCA
Short- miR-30d-3p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGCTTTCAGTCA GATGTTTGC
RT6- miR-138-5p	TGTCAGGCAACCGTATTCACCGTGAGTGGTCGGCCT
Short- miR-138-5p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGAGCTGGTGT GTGAATCAGG
RT6- miR-23a-5p	TGTCAGGCAACCGTATTCACCGTGAGTGGTAAATCC
Short- miR-23a-5p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGGGGGTTCCTG GGGATGGGA
RT6- miR-561-5p	TGTCAGGCAACCGTATTCACCGTGAGTGGTGGCAAA
Short- miR-561-5p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGATCAAGGATC TTAAACTTT
RT6- miR-27a-3p	TGTCAGGCAACCGTATTCACCGTGAGTGGTGCGGAA
Short- miR-27a-3p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGTTCACAGTGG CTAAGTTC
RT6- miR-141-3p	TGTCAGGCAACCGTATTCACCGTGAGTGGTCCATCT
Short- miR-141-3p -rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGTAACACTGTC TGGTAAAGA
MP Forward	TGTCAGGCAACCGTATTCACC
MP Reverse	CGTCAGATGTCCGAGTAGAGG
5S rRNA Forward	GCCCGATCTCGTCTGATCT
5S rRNA Reverse	AGCCTACAGCACCCGGTATT

4. References

1. S. U. Moon, M. H. Kang, J. H. Sung, J. W. Kim, J. O. Lee, Y. J. Kim, K. W. Lee, S. M. Bang, J. S. Lee and J. H. Kim, *Oncol. Rep.*, 2015, **33**, 185-192.