Supplementary Materials for

Bioengineered miRNA-1291 prodrug therapy in pancreatic cancer cells and patient-derived xenograft mouse models

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Table S1

Supplementary Materials and Methods

Fig. S1. Luciferase report assays support the action of miR-1291 on ARID3B 3'UTR. ARID3B 3'UTR-luciferase activities were significantly increased in cells treated with miR-1291 expressing plasmid (A), whereas decreased by miR-1291 antagomir (B). PANC-1 cells were co-transfected with ARID3B-3'UTR luciferase reporter plasmid (psiCHECK-ARID3B-3'UTR) and miR-1291 expression plasmid (pCMV-miR-1291), miR-1291 antagomir or their corresponding controls. Luciferase activities were determined at 48 h post-transfection. Values are mean ± SD (N = 3). *$P < 0.05$, compared to the control (unpaired Student's t-test).

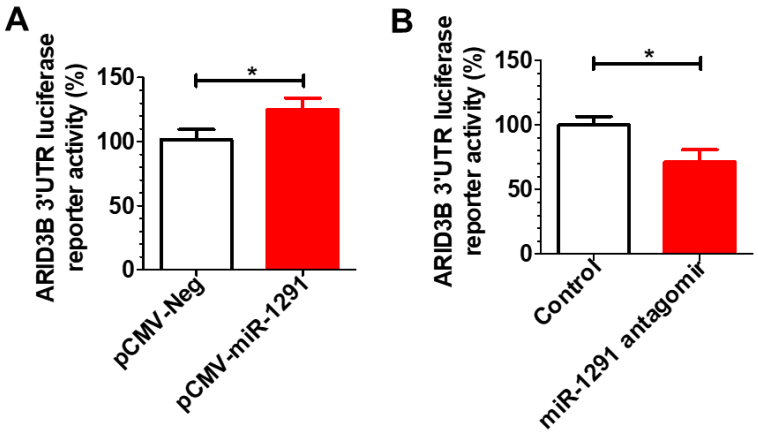


Fig. S2. ARID3B protein levels were elevated by miR-1291 prodrug in a time-dependent manner in PC cell lines. Western blot analyses showed that ARID3B protein was upregulated after transfection with miR-129 prodrug in PANC-1 (72 h post-transfection) and AsPC-1 (48 and 72 h) cells, as compared to vehicle and MSA controls. β -actin was used as a loading control.

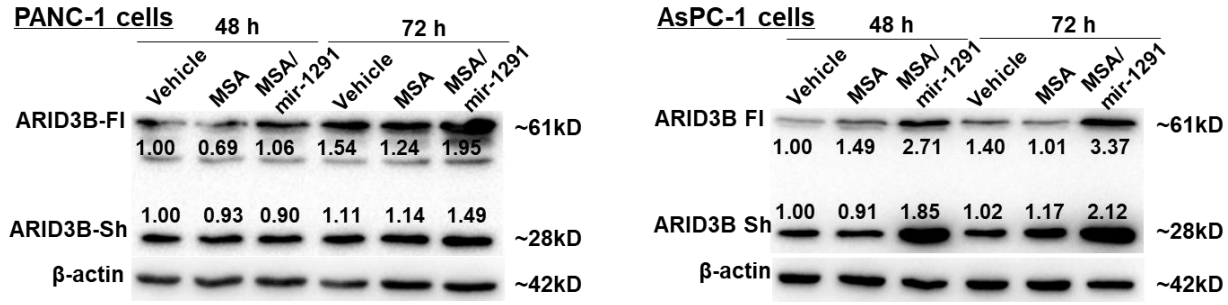


Fig. S3. Immunofluorescence of another apoptosis marker, cleaved caspase-7, in PANC-1 cells. Compared to the control, miR-1291 or Gem-nP treatment alone led to an increase of cleaved caspase-7 (c-caspase-7) levels, and combination treatment (combo) induced apoptosis to the greatest degree. Scale bar indicates 20 μm.

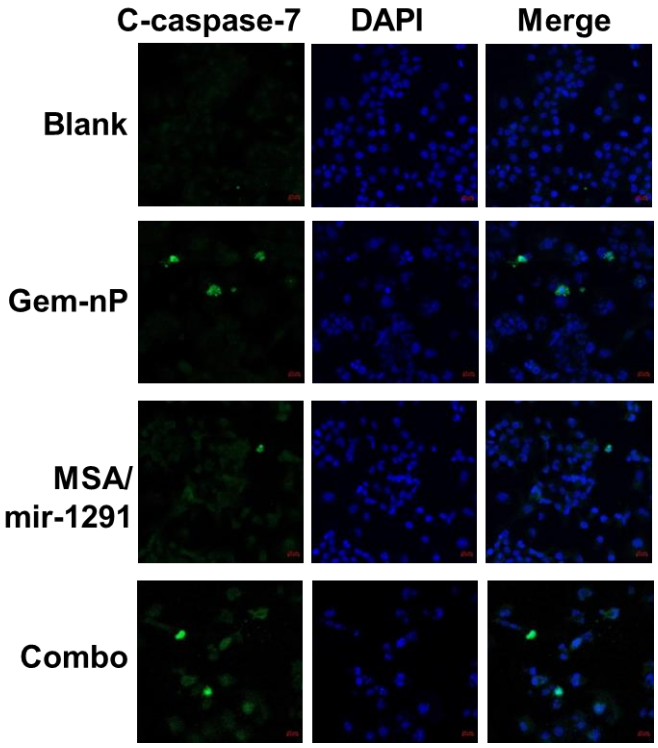


Fig. S4. **Immunofluorescence study on the actions of individual and combined drug effects on DNA damage (γH2A.X; A), mitosis inhibition (H3PS10; B), and apoptosis markers (cleaved caspase-3/7; C) in AsPC-1 cells.** Combination treatment (Combo) with miR-1291 prodrug and Gem-nP induced a higher level of cleaved caspase-3/7(C-caspase3/7), and γH2A.X than single drug treatment or blank control. Scale bar indicates 20 μm.

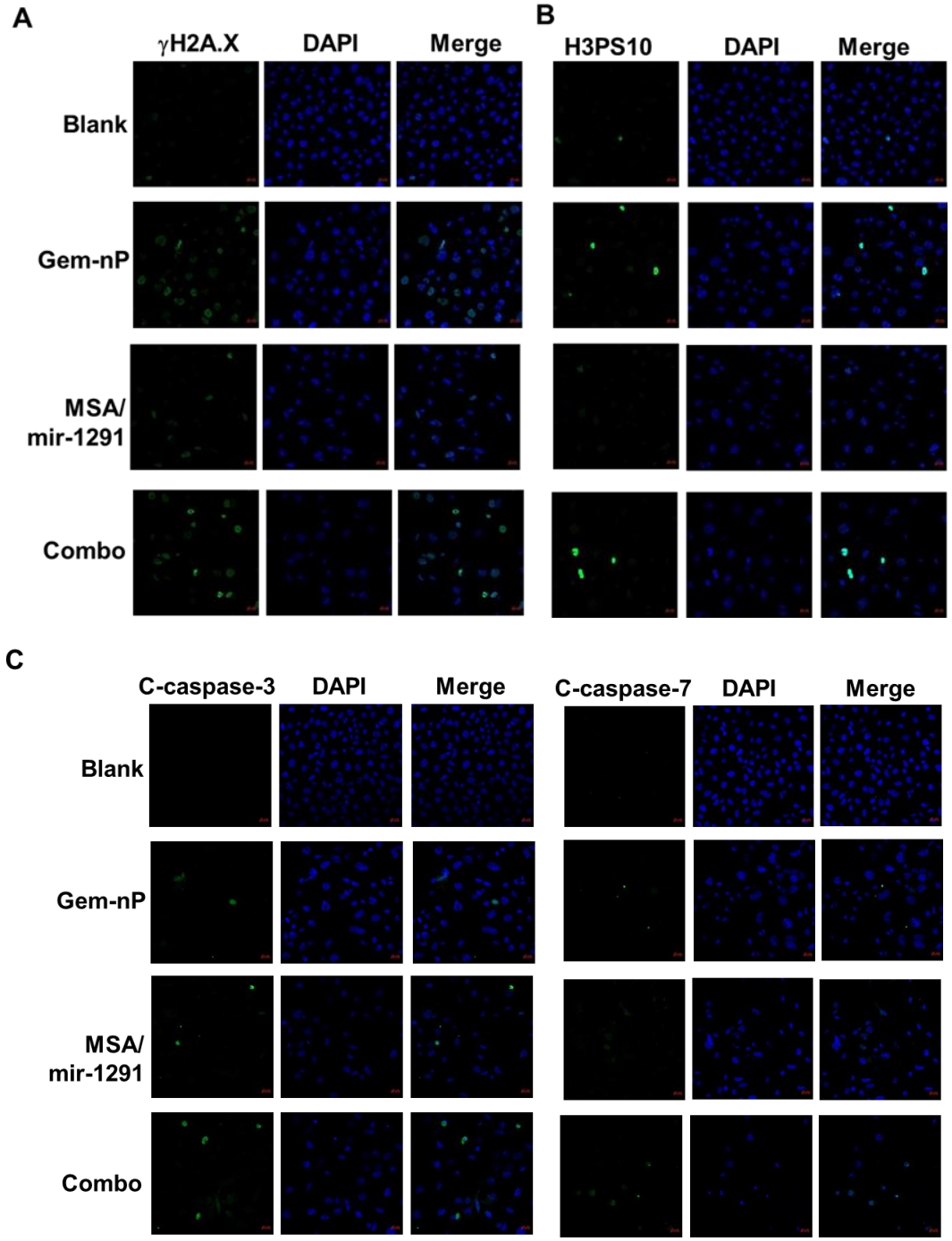


Fig. S5. *In vivo*-jetPEI-formulated miR-1291 prodrug is distributable to PANC-1 xenograft and PDX tumors in mouse models. Tumor-bearing mice were treated intravenously with a single dose of *in vivo*-jetPEI-formulated miR-1291 prodrug (25 μg/mouse). 24-h later, tumor and liver tissues were harvested and total RNAs were isolated. Levels of miR-1291 were determined with TaqMan stem-loop RT-qPCR assay, which were much higher in livers and tumors isolated from MSA/mir-1291 treated PANC-1 xenograft (A) and PDX (B) mice, as compared with MSA control (N = 2 mice per group in PANC-1 xenograft model, and N = 3 in PDX model).

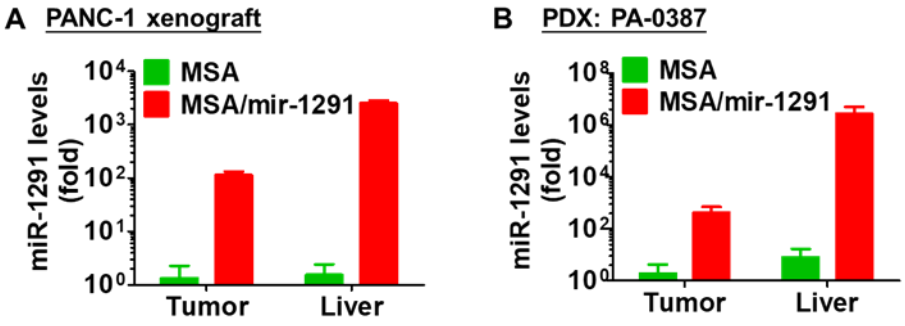


Fig. S6. Representative H&E images of PDX-PA-0387 tumors. There is not any remarkable difference in the morphology of survived tumors between different treatment groups. Scale bar indicates 100μm.

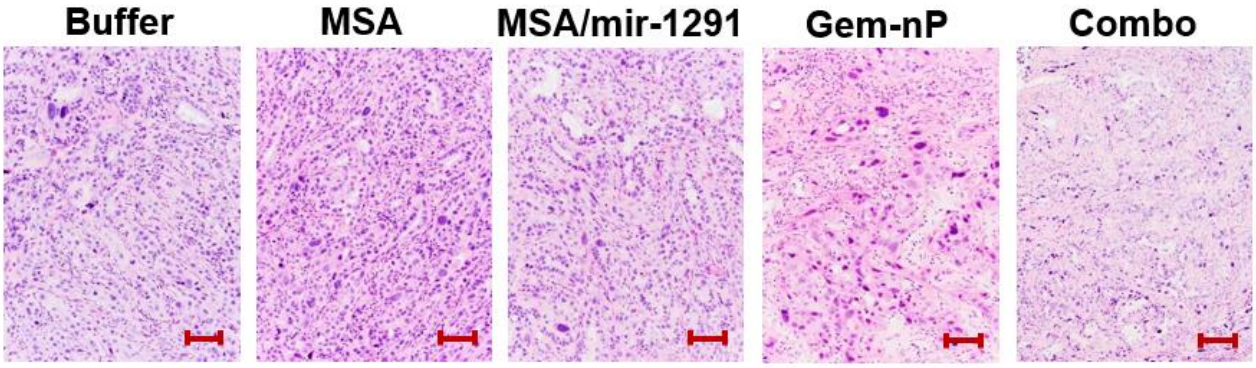
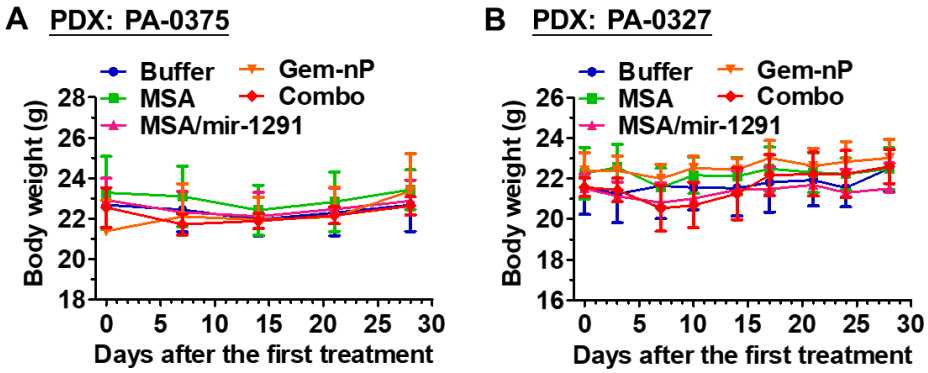


Fig. S7. miR-1291 prodrug monotherapy and combination therapy are tolerated in PDX mouse models. Body weights of the mice showed no difference between different treatments.



Supplementary Table S1. Antibodies used for Western blots and immunofluorescence analyses.

Antibodies	Manufacture	Catalog No.
Anti-ARID3B	Abcam	ab92328
Anti-γH2A.X	Cell Signaling	2577
Anti-cleaved Caspase-7	Cell signaling	9491
Anti-cleaved Caspase-3	Cell signaling	9579
Histone H3 (phospho S10)	Abcam	ab5176
Anti-β-actin	Sigma-Aldrich	A5441
Peroxidase-conjugated goat anti-rabbit IgG	Jackson ImmunoReseach	111-035-003
Anti-mouse IgG, HRP-linked Antibody	Cell signaling	7076
Alexa Fluor® 488-conjugated goat anti-rabbit IgG	Cell signaling	4412

Supplementary Materials and Methods

Materials

RPMI 1640 medium, Dulbecco's modified Eagle medium (DMEM), Fetal bovine serum (FBS), trypsin, Lipofectamine 3000 and Trizol reagent were purchased from Life Technologies (Carlsbad, CA). Gemcitabine hydrochloride salt was purchased from LC Laboratories (Woburn, MA). Paclitaxel albumin-stabilized nanoparticle formulation (Abraxane® for injectable suspension) was obtained from Celgene Corporation (Summit, NJ). RIPA lysis buffer and the complete protease inhibitor cocktail were bought from Sigma-Aldrich (St. Louis, MO). BCA Protein Assay Kit was bought from Thermo Scientific (Rockford, IL). ECL substrate and PVDF membrane were obtained from Bio-Rad (Hercules, CA). All other reagents were purchased from commercial sources and were of the analytical grade.

Production of biologic miR-1291 prodrug (MSA/mir-1291) and control RNA MSA

Expression of recombinant MSA/mir-1291 and control tRNA MSA was conducted as described recently [1, 2], while purification was performed with an improved anion exchange fast protein liquid chromatograph (FPLC) method [3]. In brief, MSA/mir-1291- and MSA-expressing plasmids were transformed into HST08 *E. coli* competent cells, respectively. Separation of target RNAs from total bacterial RNA was achieved on an Enrich-Q 10 × 100 column by using a NGC QUEST 10PLUS FPLC system (Bio-Rad), which was first equilibrated with Buffer A (10 mM sodium phosphate, pH 7.0) at a constant flow rate of 2.5 ml/min for 4.4 min and then a gradient elution: 64% Buffer B (Buffer A + 1 M sodium chloride, pH 7.0) for 10 min, 64-78% Buffer B for 8 min, and then 100% Buffer B for 3 min. FPLC traces were monitored at 260/280 nm using a UV/Vis detector. After the confirmation of target RNA by urea-PAGE analyses, fractions were pooled, precipitated by ethanol, desalted and concentrated/dissolved in nuclease-free water with an Amicon ultra-2 mL centrifugal filter (30 kDa; EMD Millipore, Billerica, MA). RNA purities were verified by a high performance liquid chromatography (HPLC) assay [1], and recombinant RNAs over 97% pure were used in this study.

Plasmids construction and luciferase reporter gene assay

The 3'UTR segment (0-972 bp from stop codon) of human ARID3B containing the predicted hsa-miR-1291 response elements (MREs; Fig. 1A) was amplified from human genome by PCR with the following primers: forward: 5'-CCG CTC GAG GTC CGT CTG TCC AGG CTC CAT TCA GGT CCT GCT G-3', reverse: 5'-TTG CGG CCG CGG GGC CGG GTT ACC CAA TCA CTT GCT TGG CTT T-3', and then inserted downstream of Renilla luciferase gene within psiCHECK-2 vector (Promega, Madison, WI) at XhoI and NotI restriction sites. Sequence was confirmed by DNA sequencing.

Luciferase reporter assay was conducted as previously reported [4]. Briefly, HEK293,AsPC-1 or PANC-1 cells were co-transfected with ARID3B-3'UTR luciferase reporter plasmids (psiCHECK-ARID3B-3'UTR) or psiCHECK-2 empty vector (0.1 μg) plus MSA or MSA/mir-1291 (0, 5, 20 nM), or miR-1291-expressing plasmid or control vector [5], or miR-1291 antagomir or control oligo using Lipofectamine 3000. 48 h post-transfection, luciferase activities were determined by a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) using a SpectraMax® M3 microplate reader (Molecular Devices, Sunnyvale, CA). Activity of Renilla luciferase was normalized to firefly luciferase and then calculated as a percentage of corresponding control.

Cell culture and treatments

AsPC-1, PANC-1, and HEK293 cells were obtained from ATCC and maintained in RPMI or DMEM media containing 10% fetal bovine serum (Gibco) at 37 ℃ with 5% CO_2 in a humidified incubator. For cell viability assays, cells were seeded in 96-well plates at a density of 5,000 cells/well, incubated overnight, and then transfected with MSA or MSA/mir-1291 (1 nM for AsPC-1 cells, 5 nM for PANC-1 cells) in the presence of various concentrations of gemcitabine (Gem, 0-10 μM) plus nab-paclitaxel (at a fixed ratio Gem/nP = 8/1). Forty-eight hours later, the viability of cells was determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). Pharmacodynamic parameters were estimated by fitting the data to an inhibitory dose-response model with variable slope, Y = Bottom + (Top - Bottom) / (1 + 10^((LogIC50-X) * HillSlope)).

RNA isolation and reverse transcription quantitative real-time PCR (RT-qPCR)

PANC-1 or AsPC-1 cells seeded in 24-well plates were transfected with MSA/mir-1291, control MSA, or vehicle using Lipofectamine 3000. Total RNA was extracted at 48 h and 72 h post-transfection using a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA), and reverse-transcribed with NxGen M-MuLV reverse transcriptase (Lucigen, Middleton, WI). RT-qPCR was performed on a CFX96 Touch real-time PCR system (Bio-Rad, Hercules, CA) by using TaqMan small RNA assay kit (Thermo Fisher Scientific) for mature miR-1291, or gene-specific primers for U6 (Forward: 5'-CTC GCT TCG GCA GCA CA-3', Reverse: 5'-AAC GCT TCA CGA ATT TGC GT-3', internal standard for miR-1291), ARID3B (Forward: 5'-GTG GCA CCC ATG TCC AAT CTA-3', Reverse: AGG ATC ACC GTC CAG TTC ATA-3'), and GAPDH (Forward: 5'-ATC ACC ATC TTC CAG GAG CGA-3', Reverse: 5'-GCT TCA CCA CCT TCT TGA TGT-3', internal standard for ARID3B). The relative expression of target gene was calculated using comparative threshold cycle (Ct) method with the formula $2^{-\Delta\Delta Ct}$.

Protein isolation and immunoblot analysis

PANC-1 and AsPC-1 cells were treated with MSA/mir-1291(10 or 20 nM for PANC-1 cells, 3 or 5 nM for AsPC-1 cells), Gem-nP (160 nM-20 nM for PANC-1 cells, 100 nM-12.5 nM for AsPC-1 cells), or the combination of MSA/mir-1291 and Gem-nP. Cells were harvested after 48 h or 72 h, and lysed with RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) whose protein concentrations were determined by a BCA kit (Thermo Fisher Scientific, Rockford, IL). Proteins (30-40 μg/lane) were separated on a 10% or 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% milk. The membranes were incubated with selective anti-ARID3B (1:1500, Abcam), anti-γH2A.X (1:1000, Cell Signaling Technology), anti-cleaved caspase-7 (1: 1000, Cell Signaling Technology), anti-histone H3 (phospho S10, 1:1000, Cell Signaling Technology) or anti-β-actin (1:5000, Sigma-Aldrich) primary antibodies, and then incubated with horseradish peroxidase-conjugated anti-rabbit (1:10000, Jackson ImmunoResearch Inc., West Grove, PA, USA) or anti-mouse (1:3000, Cell Signaling Technology) IgG (Supplementary Table S1). After washed three times, the membranes were incubated with ECL substrates, subsequently visualized and imaged by a ChemiDoc MP Imaging System (Bio-Rad). Protein band intensities were quantified by Image Lab software (Bio-Rad) and normalized to β-actin levels in corresponding samples.

Immunofluorescence

Cells were plated on 8-well chamber slides and incubated overnight for attachment. Then the cells were treated with regular medium (blank), MSA/mir-1291, Gem-nP, or the combination of MSA/mir-1291 with Gem-nP. After 48-h incubation, the medium was removed, and cells were fixed with 4% paraformaldehyde, permeabilized and blocked by 5% BSA supplemented with 0.5% Triton X-100. The blocked cells were then incubated overnight at 4℃ with a primary antibody, anti-cleaved-caspase-3, anti-cleaved-caspase-7, anti-γH2A.X, or anti-H3PS10, followed by the incubation with fluorescent secondary antibody, anti-rabbit IgG Alexa Fluor® 488 Conjugate (1:500, #4412, Cell signaling Technology). DAPI (#8961, Cell signaling Technology) were incubated with the cells to stain nuclei. The images were obtained by using a Zeiss Axio Observer.z1 Microscope coupled to a Zeiss LSM 710 Scanning Device (Zeiss, Oberkochen, Germany).

PANC-1 xenograft mouse model

PANC-1 cells were trypsinized, resuspended in PBS, and mixed with Matrigel (BD Biosciences, San Jose, CA) in a 1:1 ratio (*v/v*). Cells (7.5×10^6) in 100 μL of PBS/Matrigel solution were injected subcutaneously into the left lower back region of the nude mice for the production of PANC-1 xenograft tumor mouse models.

Pancreatic carcinoma PDX mouse models

Fresh, de-identified surgical pancreatic carcinoma specimens were obtained from Comprehensive Cancer Center Biorepository at UC Davis. The study was approved by the Institutional Review Board of UC Davis, and all patients provided written informed consent before specimen collection. None of the patients have received preoperative chemotherapy or radiotherapy. The PDX mouse model was thus established as previously described with minor modifications [6, 7]. Briefly, patients' tumor specimens were minced into 2-3 mm^3 pieces in antibiotics-containing RPMI and implanted subcutaneously into the SCID mice (F1). When the size reaches 1 cm in dimeter, PDX was harvested, cut into 2-3 mm^3, and expanded into 4 new SCID mice (F2). Three PDX models derived from different patients, PA-0387, PA-0375, and PA-

0327, were successfully engrafted, and subsequently passaged into P3 (PA-0387, PA-0375) and P4 (PA-0327) which were used in therapy studies.

Therapy studies

The tumor-bearing mice, when tumor sizes reached 70-120 mm^3, were randomized into 5 treatment groups (5-6 mice/group). The mice were treated intravenously with buffer (group 1), MSA (10 μg/mouse, group 2), MSA/mir-1291 (10 μg/mouse, group 3), Gem (300 μg/mouse) plus nP (40 μg/mouse) (group 4), or the combination of MSA/mir-1291(10 μg/mouse) with Gem (300 μg/mouse) plus nP (40 μg/mouse) (group 5) every three days for 10 times. Body weights and tumor sizes of individual animals were monitored 1-2 times per week. Tumor size was calculated by the following formula: $V= 0.5 \times Length \times Width^2$. The animals were sacrificed on day 29 from the first treatment, and tumors were dissected and fixed with 10% formalin for histological analysis. Serum samples were also prepared for blood chemistry analyses.

Immunohistochemistry

The histological features of tumor tissues and the expression of caspase-3 and Ki-67 were determined by H&E staining and immunohistochemistry assay, respectively, as previously reported [8, 9]. In brief, the fixed tumor tissues were embedded with paraffin. The paraffin slides were stained with anti-cleaved-caspase-3, anti-Ki-67 antibody or hematoxylin and eosin (H&E), and then photographed by using an Olympus camera (DP25) and CellSens software (Olympus, Center Valley, PA).

Blood chemistry profiles

Blood chemistry profiles were determined in the Comparative Pathology Laboratory at UC Davis.

Statistical analysis

Values are mean ± standard deviation (SD). According to the numbers of groups and variants, data were analyzed by Student's t-test, 1-way or 2-way ANOVA using GraphPad Prism. Difference was considered as significant when P value was less than 0.05 ($P < 0.05$).

References

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