Restoration of miR-340 controls pancreatic cancer cell *CD47* expression to promote macrophage phagocytosis and enhance antitumor immunity

Qing Xi[1,#], Jieyou Zhang[1,#], Guangze Yang[1,#], Lijuan Zhang[1], Ying Chen[2], Chengzhi Wang[1], Zimu Zhang[1], Xiangdong Guo[1], Jingyi Zhao[2], Zhenyi Xue[1], Yan Li[1], Qi Zhang[3], Yurong Da[1], Li Liu[4], Zhi Yao[1] and Rongxin Zhang[2, 1,*]

Supplemental Materials and Methods

Small interfering RNAs and transfection

The mouse miR-340 mimics and control-mimics were purchased from RiboBio (Guangzhou, China). The cells were treated with mimics (final concentration, 25 nM) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Lentiviral constructs and infection

MiR-340 overexpression lentivirus vector pGLV3-H1-miR-340-GFP-Puro (GenePharma) or pGLV3-H1-GFP-Puro empty lentiviral vector (miR-NC) (GenePharma) were transfected into HEK293T cells with the packaging vectors psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259) using PEI (Polyscience). After 48 h and 72 h, the culture medium was collected and centrifuged at 2000 × g for 5 min to remove cell debris. The supernatant was filtered; PEG 8000 was added into the supernatant, then incubated overnight at 4°C with shaking. The next day, the samples were centrifuged at 4000 rpm/min for 30 min at 4°C and resuspended for lentivirus precipitation. The Panc02 cells were infected with lentivirus collected above and selected by puromycin.

Quantitative real-time PCR

The total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA), and cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen, USA). The primers were synthesized at Genewiz (Suzhou, China). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green mix (DBI Bioscience, Germany). The reactions were performed in triplicate on an ABI PRISM

7500 Fast Real Time PCR System (Applied Biosystems Inc., USA). The fold changes were calculated using the $2^{-\triangle\triangle Ct}$. The following primers were used: miR-340 primers: 5'-GCCGTTATAAAGCAATGAGA-3' and 5'- GTGCAGGGTCCGAGGT -3'; *CD47* primers: 5'-TGCGGTTCAGCTCAACTACTG -3' and 5'- GCTTTGCGCCTCCACATTAC -3'; *GAPDH* primers :5'-CCATGTTTGTGATGGGTGTGAACCA -3' and 5'-ACCAGTGGATGCAGGGATGATGTTC -3'.

Western blot analysis

Whole cell lysates were prepared using RIPA lysis buffer in the presence of 1% phosphatase inhibitor cocktail and 1mM PMSF. The protein was subjected to SDS-PAGE after boiling for 10 min in 1 × SDS loading buffer. Proteins were electrophoresed at 80 V for 30 min and followed by 110V for 90 min, then transferred to a PVDF membrane (Millipore, USA) at 180 mA for 90 min. After blocked with 5% nonfat milk at room temperature for 1 h, the membranes were incubated with the primary antibodies overnight at 4°C. The anti-*GAPDH* antibody was purchased from Sungene (China, 1:1000). The anti-*CD47* antibody was purchased from Abclonal (China, 1:1000). After incubating with horseradish peroxidase-conjugated secondary antibody (CST, USA, 1:2000), Immunoreactive bands were visualized using the ECL Western Blotting Detection System (Millipore, USA).

Cell apoptosis and proliferation assays

For the analysis of apoptosis, cancer cells were double-stained with Annexin V-APC Apoptosis Analysis Kit (Sungene, China). In detail, the cells were resuspended in 100 μL buffer with 5 μL annexin V-APC for 10 min and then incubated with 5μL 7-AAD or PI for 5min at room temperature in the dark. A total of 500μL buffer was added, and the cells were immediately analyzed using FACSCanto II flow cytometer (BD, USA). A total of 10,000 cells per sample were acquired. For the analysis of proliferation, the cells were seeded in 96-well culture plates (2000 cells per well) and incubated for appropriate time. 10 μL Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) solution were added to the medium, and cells were incubated for 4 h in a humidified atmosphere with 5% CO_2, the absorbance at 450 nm were measured using a microplate reader.

Wound healing assay

The cancer cells were seeded into 6-well plates and cultured in DMEM containing 10% FBS. After reaching approximately 95% confluence, linear scratches were made using a 10μL micropipette tip. The cells were further cultured in DMEM containing 1% FBS. Wound width was photographed using optical microscope (20×) at 0 h and 24 h. To evaluate wound closure, three randomly selected points along each wound were marked. The measurements were obtained by measuring the distance between the wound edges using Image Pro Plus 6.0 software.

Assessment of cell proliferation using VitroGel 3D

2×10^4 miR-NC or miR-340 overexpression Panc02 cells were plated on a semi-solid polymer called VitroGel 3D (TheWell Bioscience, USA). The working solution of VitroGel 3D in DMEM was made by mixing VitroGel 3D: PBS at 1:3 ratio, then mixed with 10% FBS DMEM at 4:1 ratio. The cells in VitroGel 3D were added into a 96-well plate at 100 μL/well; plate was incubated for 20 minutes at 37 °C to turn into hydrogels quickly, then 100 μL of 10% FBS DMEM media was added on top of the VitroGel 3D. The ratios used here allow VitroGel 3D to be kept in a semi-solid state. 10 μL CCK-8 solution were added into the medium, and the cells were incubated for 4 h in a humidified atmosphere with 5% CO_2, finally measured at 450 nm with a microplate reader.

3D invasion assay and 3D migration assay

For 3D invasion assay, the 24-well cell culture inserts (Merck Millipore) were coated with 100 μL working solution of VitroGel 3D and incubated at 37°C for 20 minutes to form hydrogels, 600μL DMEM medium with 30% FBS were added into the basal chamber of the unit. 2×10^5 miR-NC or miR-340 overexpression Panc02 cells were suspended in 0.1% FBS medium and plated on top of the hydrogels in the upper chamber and incubated for 48 h at 37°C, then use a cotton swab to remove cells in the upper chamber, and dyed with crystal violet for analysis. For 3D Migration assay, the migration assay was performed in a similar way as the 3D invasion assay except that the 24-well cell culture inserts were not coated with VitroGel 3D.

Additional Figures and Legends:

Figure S1: The gating plan of phagocytosis in FACS plots.

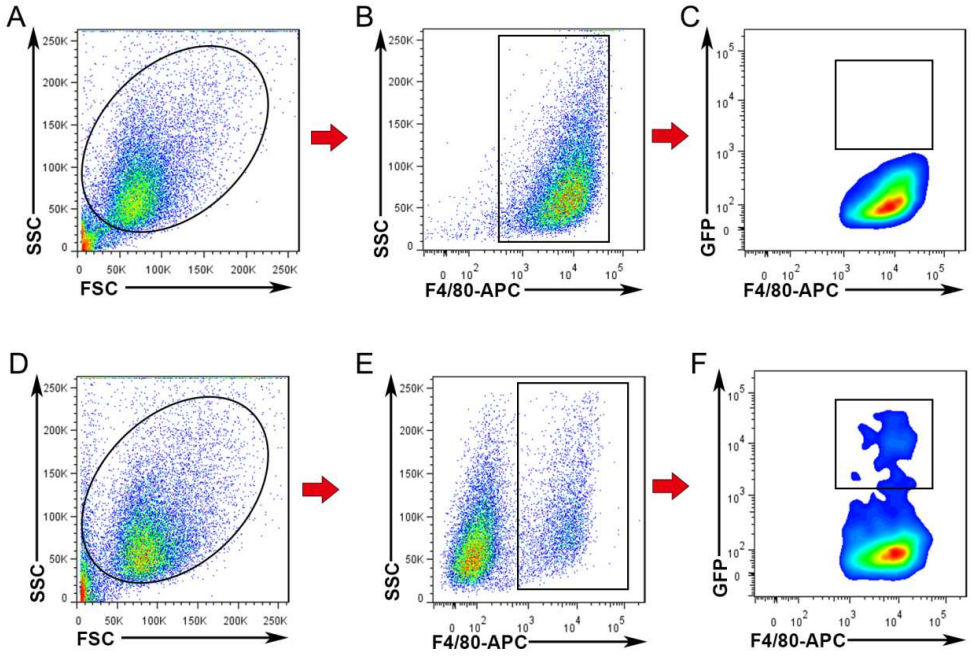

Figure S1: In the FACS plots, the macrophages ($F4/80^+$) were selected firstly, then the macrophages engulfed GFP^+ cancer cells ($F4/80^+GFP^+$) were marked. (A-C) The gating of phagocytosis in macrophages alone. (D-F) The gating of phagocytosis in macrophage co-cultured with pancreatic cancer cells.

Figure S2: Representative images of phagocytosis of tumor cells by macrophages in immunofluorescence staining.

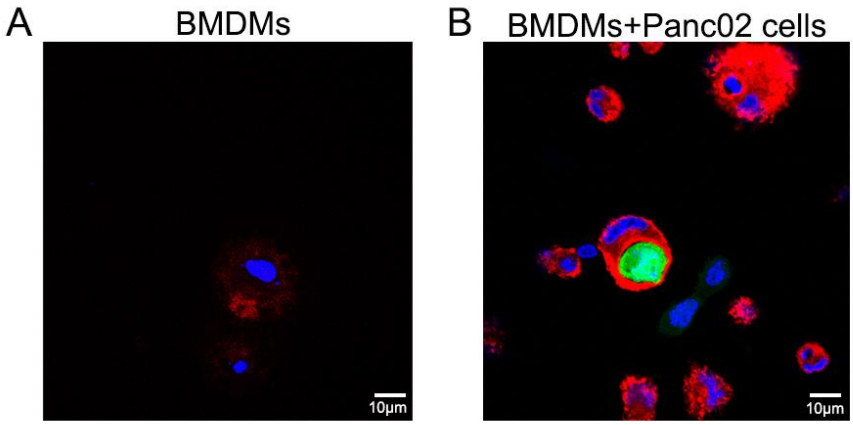

Figure S2. A: Representative image of BMDMs alone. B: Representative image of phagocytosis of Panc02 cells by macrophages in co-culture system, the phagocytosis was inspected using a Fluoview FV1000 Laser Scanning Confocal Microscope. Magnification:100×. Macrophages were stained in red ($F4/80^+$), cancer cells were green (GFP^+), and nuclei were blue ($DAPI^+$).