**Supplementary Materials**

**Thy1-Targeted Microbubbles for Ultrasound Molecular Imaging of Pancreatic Ductal Adenocarcinoma**

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**Supplementary Methods**

**Engineering of an Thy1-targeted single-chain antibody (Thy1-scFv)**

A scFv yeast surface display (YSD) library (2.5 x 109 diversity) (**Fig. 1B**), in which EBY100 yeast cells were transformed with pCT surface display vector containing the scFv gene, was screened with a combination of magnetic bead sorting and fluorescence-activated cell sorting (FACS) with recombinant human and murine Thy1 as described ([1](#_ENREF_1)). A single round of yeast isolation consisted of two isolations with streptavidin-magnetic beads (ThermoFisher Scientific) conjugated to biotinylated recombinant human and murine Thy1 (B-Thy1, Abcam) and one FACS isolation against double-positive yeasts with 500 nM Thy1 and c-Myc. Then, scFv gene fragments were obtained after error-prone polymerase chain reaction (E-PCR) against plasmids isolated from sorted yeasts and new libraries for the next rounds of yeast isolation were constructed by transformation of the fragments and pCT vector, as described ([1](#_ENREF_1)). Seven rounds of yeast isolation were performed to complete the screening. After the fourth round of yeast isolation, yeasts were further isolated after double staining with lower concentration of Thy1 and an anti-cMyc antibody (eBioscience) using FACS. For FACS isolation, yeasts were stained with 50 nM B-Thy1 at 4οC for 1.5 hours. After a simple wash with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (PBSA), yeasts were incubated with streptavidin-APC (1:100 dilution) and chicken anti-cMyc (1:100 dilution) antibodies at 4°C for 1 hour. Yeasts were then counterstained with Alexa 555-conjugated goat anti-chicken IgY (1:100 dilution) at 4°C for 40 min. Yeasts of the highest Thy1-binding affinity were isolated with FACS and further cultured in 10 mL of SD-CAA media at 30°C and 250 rpm for 1 day. After the induction of scFv-YSD, isolated yeast cultures were centrifuged at 5000 rpm for 1 min and placed into SG-CAA media. At final seventh round, the FACS isolations were repeated twice after staining with 10 nM, 5 nM, 3 nM and 1 nM Thy1. The final isolated yeast clone (Thy1-scFv) with the highest affinity was further analyzed, and the plasmid was isolated with a Zymoprep Yeast Plasmid Miniprep II kit. The isolated plasmid of the yeast clone Thy1-scFv was sequenced and subcloned into E. coli.

**Purification of Thy1-scFv**

To construct the scFv expression vector for Thy1-scFv, the gene was amplified. The amplified PCR fragment was digested with NcoI and XhoI and ligated into the same sites in the E. coli pET32b expression vector, which contains a 6xHis tag and enterokinase sequence in the N-terminus of Thy1-scFv. The scFv ligand Thy1-scFv purification was performed in SHuffle T7 E. coli, transformed with bacterial expression vector. A single bacterial colony grown on an LB plate supplemented with ampicillin (50 μg/mL) was inoculated into 5 mL of lysogeny broth (LB) media supplemented with ampicillin. After overnight culture, bacteria were transferred to 1 L of lysogeny broth (LB) media and grown at 30°C and 250 rpm for 4 hours. Bacteria were further cultured at 30°C and 250 rpm for 6 hours after 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. The bacterial pellet was harvested via centrifugation at 3,200 x g for 10 min, and was resuspended in 3 mL of ice-cold lysis buffer. The supernatant obtained by centrifugation at 12,000 x g for 5 min were applied to a HisTrap FF column (GE Healthcare Biosciences, PA) in an AKTA FPLC system (GE Healthcare Biosciences), and 6xHis-tag Thy1-scFvs were isolated and then lyophilized. The concentration of purified Thy1-scFv protein was measured by UV spectrometry after dissolving in PBS. Evaluation of purity of the Thy1-scFv was analyzed using SDS-PAGE electrophoresis. 30µl of each purified protein and 6µl of 5X reducing SDS loading buffer were added to 1.5 ml tubes and denatured at 96˚C for 5 minutes. The samples were run on SDS-PAGE gel in SDS running buffer at 30 mA for 2 hours. The gel was then stained with Coomassie Brilliant Blue for 1 hour and 3 subsequently destained for at least 12 hours with Coomassie destaining solution. The gel was visualized and analyzed using a BioRad Gel-Doc system.

**Preparation of Thy1-targeted microbubbles**

For this proof-of-principle study, commercially available perfluorocarbon-filled, lipid-shelled, streptavidin-coated contrast microbubbles (**Fig. 1C**) (MicroMarker, VisualSonics, Toronto, Canada) with a mean diameter of 1.5 μm (range, 1-3 μm) were reconstituted in 1 mL sterile saline (0.9% sodium chloride) according to the manufacturer’s protocol ([2](#_ENREF_2),[3](#_ENREF_3)). Three types of Thy1-targeted microbubbles were prepared: Microbubbles targeted to both human and murine Thy1 (MBThy1-scFv) using the new Thy1-scFv; and two positive control microbubbles (MBThy1) targeted either to murine Thy1 (for imaging of transgenic mice) or to human Thy1 (for human xenograft imaging) using commercially available antibodies as binding ligands (mouse anti-human, eBioscience; San Diego, CA; or rat anti-mouse Thy1 monoclonal antibodies, eBioscience). Six µg of either biotinylated Thy1-scFv or biotinylated antibodies were mixed with 5x107 microbubble for 10 min at room temperature. As negative control microbubbles, non-targeted microbubble (MBNon-targeted) and biotinylated scrambled scFv coupled to microbubbles (MBscFv-scrambled) were used. Non-bound ligands were removed by centrifugation at 300g for 2 min. The average number of attached ligands per square micrometer of the microbubble surface was approximately 7,600 for all microbubble types ([3](#_ENREF_3),[4](#_ENREF_4)).

**Cell culture flow chamber cell attachment studies of scFv-conjugated microbubbles**

Binding specificity of MBThy1-scFv to the target Thy1 was also assessed in cell culture experiments under flow shear stress conditions simulating flow in blood capillaries by using a parallel flow chamber experimental set-up (**Fig. 1D**). MS1Thy1 cells between passages 2 and 4 were used in all experiments. Before flow chamber experiment, FACS analysis was performed in order to confirm Thy1 expression of MS1Thy1 cells. The geometric mean fluorescence intensity was determined using FlowJo software. MS1Thy1 cells were grown on coated (Sigmacote; Sigma, St Louis, Mo) neutral-charged glass microscope slides (VWR, USA) for 24 hours and mounted on a parallel plate flow chamber (GlycoTech, Rockville, Md). A syringe infusion and withdrawal pump (Genie Plus; Kent Scientific, Torrington, Conn) was used to maintain the flow rate of 0.6 mL/min, corresponding to a wall shear stress rate of 100 sec-1, similar to that in tumor capillaries ([5](#_ENREF_5)). Flow chamber cell attachment studies were performed as described ([4](#_ENREF_4)). The glass microscope slides were inverted and positioned in the parallel flow chamber apparatus in order to allow microbubbles to float and then bind to Thy1-expressing cells. In brief, solutions were passed over cells in the following order: PBS for 2 minutes; 5 x 107 of either MBThy1, MBThy1-scFv, MBscFv-scrambled, or MBNon-targeted in PBS for 4 minutes; and finally washing with PBS for 2 minutes. The adhered microbubble number on the MS1Thy1 cells monolayer was quantified manually by counting attached microbubbles on MS1Thy1 cells with a phase-contrast bright-field microscope (Axiovert 25; Carl Zeiss, Thornwood, NY; original magnification, x 100) to assess the number of attached microbubbles per cell. At least five random fields of view of these slides were immediately imaged. Note that microbubbles can be visualized as small, rounded particles and were considered to be attached to MS1Thy1 cells when there was direct contact with the cells without free floating. Blocking studies were performed to further confirm binding specificity of MBThy1-scFv to Thy1. For this purpose, MS1Thy1 cells were incubated with 100 μg/mL purified Thy1-scFv in PBS for 30 min at 37°C before flow chamber cell attachment study to block the Thy1 receptor. All flow chamber experiments were performed in triplicate.

***Ex vivo* analysis of pancreas tissues**

Immediately following the imaging sessions, *in vivo* fixation of the pancreas via intracardiac perfusion was performed to minimize rapid self-digestion of the pancreas. Perfusion was performed under deep anesthesia with 4% paraformaldehyde at a rate of 4 mL/min for 5 min, in accordance with animal care guidelines (**Fig. 1F**). The tumors were then excised, fixed in 4% paraformaldehyde at 4°C for 24 hours and then cryoprotected by incubation in a 30% sucrose solution at 4°C for another 24 hours. Tumors were embedded in optimum cutting temperature compound (O.C.T.; Sakura Finetek), frozen on dry ice, and sectioned to 5-10 μm thickness.

Orthotopic human PDAC xenografts sections were incubated in 5% normal goat serum in PBS for 30 min to block nonspecific proteins. Sections were simultaneously incubated for 12 hours (4°C) with 1:100 each of primary antibodies: rabbit anti-human Thy1 antibody (Sigma) and rat anti-mouse CD31 (BD Pharmingen). Secondary antibodies (goat anti-rabbit Alexa Fluor 488 antibody and donkey anti-rat Alexa Fluor 594 antibody; Invitrogen) were simultaneously applied at 1:600 dilutions in PBS for 30 min at room temperature to confirm Thy1 expression on the tumor neovasculature.

Transgenic tumor sections, sections from normal pancreatic and chronic pancreatitis tissues were blocked in 3% bovine serum albumin with 3% goat and 3% donkey serum in PBS for 30 minutes and incubated with rat anti-mouse Thy1.2 (eBioscience) and rabbit anti-mouse CD31 (Abcam, Cambridge, MA) overnight at 4°C. Secondary antibodies (donkey anti-rabbit Alexa Fluor 488 antibody and donkey anti-rat Alexa Fluor 546 antibody, both 1:250; Invitrogen) were incubated for 30 min at room temperature. All samples were mounted using ProLong Gold (Invitrogen Life Technologies). Fluorescent images were acquired by microscopy at a magnification of 100X (Axiovert 25; Carl Zeiss, Germany). Additional tumor slides were stained with hematoxylin eosin and scanned using a Nanozoomer (10X magnification, Hamamatsu, Japan). To further confirm that MBThy1-scFv bound within the neovasculature of PDAC with direct microscopic visualization, MBThy1-scFv was fluorescently labeled and injected intravenously in an additional two orthotopic human PDAC xenograft models as well as two transgenic mice with PDAC. In brief, MBThy1-scFv were fluorescently labeled with CellMask Green (ThermoFisher, Waltham, MA) according to manufacturer instructions. The labeled microbubbles were washed twice in 0.9% saline, centrifuged at 200 RPM for 5 minutes, resuspended in 100 μl of 0.9% saline, and injected intravenously along with 50 µg of Lectin DyLight 594 (Vector Laboratories, Burlingame, CA) into mice. Microbubbles were allowed to adhere for 4 minutes. To washout all non-attached microbubbles, mice under deep anesthesia were perfused with 4% paraformaldehyde at a rate of 4 mL/min for 5 min, in accordance with animal care guidelines. The pancreas was then harvested and fixed in paraformaldehyde at 4°C overnight. The following day, the pancreas was placed into cryopreservation (30% sucrose in water) for 24 hours at 4°C. Pancreas were then embedded in an optimal cutting temperature medium (Fisher Scientific), frozen, and tumors were cut into 20-µm-thick slices on a cryomicrotome. Fluorescent images were acquired by microscopy at a magnification of 20X (Axiovert 25; Carl Zeiss, Germany).

**Statistical Analysis**

Data were presented as mean ± standard deviation. The two-sample Wilcoxon test was used for pair-wise comparisons of measurements in flow chamber experiments. For comparison between MBThy1-scFv and MBThy1 versus MBscFv-scrambled, or MBNon-targeted in the same tumor and for competition assays, the one-sample paired Wilcoxon test was used. For assessing associations between Thy1-expression levels and cell attachment, Spearman’s correlation coefficient was estimated and the corresponding P-value was obtained based on Fisher’s transformation using commercially available software (IBM SPSS statistics software, version 20; IBM Corp, Chicago, IL). The significance level was set at 0.05.

**References**

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