**A Virus-Infected, Reprogrammed Somatic cell-derived Tumor cell (VIReST) regime can prevent initiation and progression of pancreatic cancer.**

**Running Title: Stem-cell-based prophylactic vaccination for pancreatic cancer**

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**Supplementary Methods**

**Blastocyst injection**

Blastocyst injection was conducted in the facilities of GIBH, China. For generation of chimeras, iPSCs were injected into ICR blastocysts using a Piezo Micro Manipulator. Injected blastocysts were transplanted into pseudopregnant ICR females for generation of viable offspring.

**Teratoma Formation and Histological Analysis**

iPS cells（WT; KPC; WT-KP) were suspended at 5X107 cells/ml in PBS and 100µl of the cell suspension (5 X106 cells) was injected subcutaneously into the dorsal flank of nude mice. Two to four weeks after the injection, tumors with diameter below 1.5cm were surgically dissected and fixed in PBS containing 4% formaldehyde, and then embedded in paraffin. Sections were stained with hematoxylin and eosin. Pictures of various tissues that are present in the teratoma were taken by Olympus microscope under the guidance of a pathologist.

**Western blotting**

2x105 cells were lysed on ice using RIPA containing 50 mMTris-HCl (pH 7.4), 150 mMNaCl, 1% NP-40, 0.1% SDS and 1mM PMSF. Protein concentration in samples was determined by Bradford assay. Samples were separated on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane using a semi-dry transfer system (Biorad). Membranes were blocked in 0.1% Tween 20 in PBS supplemented with 5% fat-free milk for 40 minutes and then incubated with primary antibodies specific to the following proteins: E1A (MS-587-P1, NeoMarkers), Vaccina virus proteins (9503-2057, Biogenesis Ltd), Pdx1 (ab47267, Abcam). Actin was used as a loading control (5125s, Cell signaling). Antibodies were diluted 1:200 – 1:1,000 in 5% fat-free milk. Membranes were transferred to the appropriate horseradish peroxidase-labelled secondary antibodies: Goat anti-mouse (1:5,000 dilution) and Goat anti-rabbit (1:5,000 dilution) (ZSGB-Bio, Beijing, China). Secondary antibodies were detected using the ECL Western Blotting Detection System.

**Cell immunofluorescence**

Cells cultured on slides were fixed with 4% PFA for 30 min at room temperature, then permeabilized and blocked (PBS containing 3% BSA/0.2% triton (1:1) mixture) for 40 min. Cells were then incubated overnight with primary antibodies at 4°C, followed by incubation with goat α-rabbit secondary antibody (AbCam: ab150077) or Rhodamine (TRITC)-conjugated AffiniPure Bovine Anti-Goat IgG (H+L) (Jackson ImmunoResearch, 805-025-180) at room temperature for 1.5h**.** Nuclei were stained with DAPI (Sigma Aldrich). Stained cells were examined using an Olympus ﬂuorescence microscope. The primary antibodies used were all obtained from AbCam unless stated; Nanog (ab80892); Oct4 (ab18976); Sox17 (ab191699); Foxa2 (ab108422); Sox9 (ab26414), Sox2 (R&D systems: AF2018), TTF1 (ab76013) and Pdx1 (ab47267). Antibodies were diluted 1:200 in 3% BSA.

**Quantitative PCR**

Total RNA was isolated using TRIzol and converted to cDNA using HiScriptTM Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, China). Quantitative (q) PCR was performed using the ABI StepOnePlusTM according to the AceQTM qPCR SYBR Green mastermix protocol (Vazyme, China). All qRT-PCR results presented in this study were from at least two independent experiments with independent sample preparations. The primers used are listed in supplementary table 2.

**Cell growth curve**

1x105 log-growth phase cells/well were cultured in 24-well plates for 6 days using DMEM containing 10% FBS. Every 24h, cells from three wells were removed using 0.25% trypsin and numbers counted under a microscope.

**Soft agar colony formation assay**

1ml DMEM containing 10% FBS and 0.75% agar was used to coat the bottom of each well of a 6-well culture plate and allowed to solidify. Cells were harvested and single-cell suspensions produced in complete culture medium at 50,000 cells/ml.  100µl of the single cell suspension was mixed with 1ml DMEM containing 10% FBS and 0.3% agar and the mixture was added to each pre-coated well. Dishes were incubated at 37°C in an incubator for 3 weeks and photographs of resulting colonies were taken. Colonies in each well were counted and averaged and the colony formation efficiency was calculated (clone number/total cell number).

**Cell cytotoxicity assay**

The cytotoxicity of the viruses in each cell line was assessed 6 days post-infection with virus using an MTS non-radioactive cell proliferation assay kit (Promega) according to the manufacturer’s instructions. Cell viability was determined by measuring absorbance at 490nm using a 96-well plate absorbance reader (DTX800, Beckman) and a dose response curve created by non-linear regression allowing determination of an EC50 value (dose required to kill 50% of cells).

**MTS detection of cell proliferation after MMC treatment**

Cells were plated at 2x104 cells/well in 96-well plate in 200µl DMEM containing 5% FCS. Six hours later cells were infected with Ad5 (MOI=50 pfu/cell for 4h) or VVL15RFP (1pfu/cell for 2h) and then treated with 200µg/ml MMC for 2h. One plate of treated cells was used in each time point. Six wells of uninfected cells and six wells of medium only were used as positive and negative controls respectively. Plates were incubated at 37°C and 24 hours later, 20µl of MTS reagent was added to the OD490 read on microplate reader (DTX880, Beckman). The same procedure was repeated at 72 hours.

**Viral replication assay**

Cells were infected with 10 PFU/cell of Ad5 or 1 PFU/cell of VVL15-RFP in DMEM containing 2% FCS for 18h. Samples including cells and supernatants were harvested in triplicate at 24-hour intervals up to 96h and frozen at -80oC, freeze-thawed three times to release virions, and titrated using JH293 cells for Ad5 or CV1 cells for VVL15-RFP. The Reed–Muench mathematical method was used to calculate the 50% tissue culture infective dose (TCID50) value for each sample (1). Viral burst titres were converted to PFU per cell based on the number of cells present at viral infection.

**IFN-**γ **detection by ELISA**

Murine spleens were isolated 21 days after treatment, mashed through 70μm BD Falcon™ cell strainers and flushed through with complete T-cell media (Roswell Park Memorial Institute (RPMI)-medium 1640 (Sigma Aldrich), 10% FCS, 1% streptomycin/ penicillin, 1% sodium pyruvate and 1% non-essential amino acids (Gibco®) and 0.1% β-mercaptoethanol). Splenocytes were re-suspended in red blood cell (RBC) lysis buffer (Sigma-Aldrich) and re-suspended in complete T-cell medium.

Cell suspensions incubated for 72 hours with mitomycin-treated cell lines or peptides (supplementary table 3) prior to assessment of IFNγ production was assessed by ELISA (eBioscience) according to manufacturer’s protocol. Supernatants were diluted 1:20 in the provided reagent.

**Histopathological examination and immunohistochemistry**

Pancreas tissues from KPC mice were immediately snap-frozen in liquid nitrogen-cooled isopentane. Samples were cut to thickness of 6μm with a Leica EG1160 microtome (Leica Microsystems UK Ltd, Milton Keynes, UK), air dried at room temperature and stained with haematoxylin and eosin (H&E) and immunohistochemistry (IHC) antibodies to detect CD4+, CD8+ or TRegcells. Antibodies utilized for IHC are listed in supplementary table 4. Images were acquired under an Olympus microscope. CD4+ T cell presence in the whole tissue slice was scored at the different grades based on the average positive cells over ten high power fields (x200). Grade 1: 0-25 positive cells/HPF; grade 2: 25-50 positive cells/HPF; Grade 3: 50-75 positive cells/HPF; Grade 4: More than 75 positive cells/HPF. The CD8+ cells was scored as the average number over ten high power fields (x200) or whole sections.

**Immunophenotyping using FACS**

Single cell suspensions of immune cells were obtained from homogenized spleens and draining lymph nodes of vaccinated and unvaccinated mice. A master mix for each immunophenotyping group was prepared in advance and all antibodies (listed in supplementary table 4) were diluted 1:150 using PBS. Immunophenotyping was performed by staining 1x106cells derived from each organ in 100µl antibody mix for 1 hour on ice. Stained cells were washed twice in PBS buffer and re-suspended in PBS buffer. Data were acquired on a MACSQuant Analyzer 10 (MiltenyiBiotec).

**Transcriptome sequencing**

KPC, KP-AC, TB11381, DT6606, TB32043 and TB32047 cell transcriptomes were sequenced in BGI. Tec, Shenzhen, China. RNA libraries were prepared using an Illumina TruSeq RNA Sample Prep Kit. Briefly, 200ng total RNA sample was purified by oligo-dT beads, then poly (A)-containing mRNA were fragmented into small pieces with Elute, Prime, Fragment mix. First-strand cDNA was generated by First Strand Master Mix and Super Script II (Invitrogen) reverse transcription. The Second Strand Master Mix was added to synthesize second-strand cDNA (16oC for 1h). The purified fragmented cDNA was combined with end-repair mix and incubated at 30oC for 30min. The end-repaired DNA was purified using Ampure XP Beads (AGENCOURT). A-Tailing Mix was then added and samples were incubated at 37oC for 30 min. These samples were incubated with RNA Index Adapter and Ligation Mix at 30oC for 10 min. The end-repaired DNA was purified using Ampure XP Beads (AGENCOURT). Several rounds of PCR amplification with a PCR Primer Cocktail and PCR Master Mix were performed to enrich the cDNA fragments. The PCR products were finally purified with Ampure XP Beads (AGENCOURT).

After the libraries were obtained, they were amplified on cBot to generate a cluster on the flowcell (TruSeq PE Cluster Kit V3–cBot–HS, Illumina). The amplified flowcell was sequenced using the HiSeq 2000 System (TruSeq SBS KIT-HS V3，Illumina) with a read length of 100 bp, producing on average 24M paired-end sequence reads per sample. Sequencing reads were aligned to the mouse genome build mm10/GRCm38 with the HISAT2 aligner (2). The number of reads uniquely aligned (mapping quality score q > 10) to the exonic region of each gene were counted using HTSeq (3), based on GENCODE version 9 mouse gene annotation. Only genes that achieved at least one count per million (CPM) mapped reads in at least one sample were included, leading to 15,687 filtered genes in total. Read counts were further normalized using the conditional quantile normalization (cqn) method (4), accounting for gene length and GC content, with the RPKM (Reads Per Kilobase of transcript per Million mapped reads) values derived for genes across the six samples. The overall gene expression correlations between samples were subsequently calculated. The data have been deposited in Gene Expression Omnibus (GEO) using the accession number GSE85230.

**Viral infection for assessment of HMGB1 production**

DT6606 cells were infected with VVL15 or Ad5 at the indicated PFU. Cells were also mock-infected or treated with 2µM of mitoxantrone (MTX). 48h after infection, supernatants were collected and Western blotting was used to detect the expression of HMGB1.

**Assessing cell surface exposure of Calreticulin by flow cytometry**

Calreticulin staining was performed in V-bottom 96-well plates. Samples were incubated at room temperature for 30 min in 75μl of FACS buffer containing anti-calreticulin primary antibody diluted at 1:150. Samples were washed and re-suspended in 100μl FACS buffer containing a secondary goat anti-rabbit – Alexa fluor® 488 (1:200). Data analysis was performed on BD FACSDIVA™ software and FlowJo V10 softwares (*Tree star, Inc*).

**ATP assays**

DT6606 cells were seeded at 1x105 cells / well in 96 well-plate in 90μl per well of DMEM and treated the following day by addition of virus in 10μl DMEM. At 48 hours post-infection extracellular ATP was measured by addition of 50µL media and 40µL Hepes buffer (25mMHepes in PBS, pH 7.4) to each well. Simultaneously, to measure intracellular ATP, 40 l of saponin buffer (containing 0.05 % saponin in Hepes buffer) were added to each well and incubated for 1 min. 90μl of the mixture was transferred into a new white bottom 96-well plate. 10μl of ATP assay mix (lyophilised powder*; Sigma*) were promptly added to the white bottom 96-well plates. Luminescence was detected using a luminometer.

**Mouse anti-nuclear antibody ELISA**

Tails were cut at 0.3-0.5cm from PBS control KPC mice, vaccinated KPC mice and vaccinated KP mice to collect blood (100µl/mouse). Blood was coagulated for 10-20 minutes at 37°C and then centrifuged for 20 minutes at 3,000 rpm to collect serum. ANA ELISA was carried out according to the protocol of the manufacturer of the mouse ANA ELISA kit (SBJ-mo134, Senbeijia Nanjing Biotechnology Co, Ltd). Absorbance at 450nm was recorded and the amount of ANA in each sample was calculated based on the standard curve.

**High resolution ultrasound on mice pancreas**

Pancreatic tumors from KPC mice at different ages were verified by high-resolution ultrasound (Vevo 2100, VisualSonics) according to a previous report (5). Briefly, mice were anesthetized using Avertin (0.45mgkg-1) and 4ml of sterile saline administered by intraperitoneal injection. Ultrasound gel was applied and the MS550D transducer was placed on the abdomen and orthogonal to the plane of the imaging platform. 3D image capture was enabled as per manufacturer’s instructions. The ultrasound software study management function was used to derive tumor volume according to the manufacturer’s instructions.

***In vivo* experiments**

All animal procedures were approved by the Animal Welfare and Research Ethics Committee of Zhengzhou University (Zhengzhou, China). Mice were housed in groups in accordance with the regulations for mouse welfare and ethics of Zhengzhou University with 12h dark-light cycles and free access to food and water.

LSL-KRasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre (KPC) mice were kindly provided by David Tuveson. Genotyping was performed using the following primers; KRas F: 5’-CCATGGCTTGAGTAAGTCTGC-3’ KRasR 5’-CGCAGACTGTAGAGCAGCG-3’ (550bp); P53\_F: 5’-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3’ P53\_R: 5’-CTTGGAGACATAGCCACACTG-3’ (270bp); Cre\_F: 5’-CTGGACTACATCTTGAGTTGC-3’ Cre R: 5’-GGTGTACGGTCAGTAAATTGG-3’ (450bp). Male C57/Black6 mice and nude mice were purchased from Vitalriver.com, Beijing, China. Female ICR mice were provided by the Guangzhou Institute of Biomedicine and Health (GIBH), Chinese Academy of Sciences, Guangzhou, China.

In subcutaneous tumor models, animals were randomly assigned to treatment groups and tumor growth was measured using electronic callipers until tumors measured 1.8cm in diameter or ulcerated, at which point the animal was sacrificed. For KPC pancreatic cancer models, animals were assigned randomly to treatment groups and animal survival was monitored by assessment of animal well-being every other day. Animal caretakers were blinded to treatment groups in all cases.

Statistical analysis was carried out using Graph Pad Prism 5 and SPSS 19.0 software. The results were represented as mean ± standard or deviation (SD) or ± standard error of the mean (SEM). Differences between groups were analysed using Students’ unpaired T tests or Kaplan–Meier survival analysis. Differences were considered statistically significant when the p < 0.05.

***In vivo* tumor challenge**

2x106 tumor cells were implanted subcutaneously into the right ﬂank of 4-6 weeks old male nude mice or KPC littermates (129J/Bl6) control male mice. The tumor growth was assessed twice weekly.

***In vivo* CD8+ or CD4+ T cell depletion**

α-CD8 (TIB2100) or α-CD4 (GK1.5) (Provided by Professor Shengdian Wang, The Chinese Academy of Sciences, Institute of Biophysics) was injected into the abdominal cavity of the mice one day before prime and two times/week in the interval between prime and boost, and four weeks after boost at 200µg/mouse/timepoint**.** CD8+ or CD4+ T cell depletion was confirmed using FACS analysis throughout the experiment.

α-PD1 treatment

200ug α-PD1 (Bioxcell) was administered to each mouse intra-peritoneally 1 week post prime and 1 week post-boost injection.

***In vivo* vaccination protocol**

TB11381, DT6606, KPC or KP-AC cells were infected with replicating Ad5 (50pfu/cell) for 4h prior to inactivation using 200µg/ml MMC for 2h at 37°C. 5x106 TB11381, KPC or KP-AC cells were delivered subcutaneously into the flank of four-week-old KPC mice and 2x106 DT6606 cells were delivered subcutaneously into the flank of C57/Bl6 or KP littermate mice as the prime stage of the vaccination protocol. Four weeks later, DT6606, KPC, KP-AC Or TB11381 cells were infected with VVL15-RFP at 1pfu/cell for 2h followed by inactivation using 200µg/ml MMC for 2h at 37°C. 5x106 (KPC, KP-AC or TB11381) or 2x106 (DT6606) were then delivered subcutaneously into the flank of primed mice as the boost stage of the vaccination protocol. Immunized C57/Bl6 mice were subsequently re-challenged two weeks after the booster immunization with injection of 6x106 DT6606 cells in the flank and tumor growth and animal survival were monitored.

**Supplementary Methods References**

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