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ARTICLE

Therapeutic activity of retroviral replicating vector-mediated prodrug activator gene therapy for pancreatic cancer

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Abstract

Toca 511, a retroviral replicating vector (RRV) encoding the yeast cytosine deaminase (yCD) prodrug activator gene, which mediates conversion of the prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU), is currently being evaluated in Phase II/III clinical trials for glioma, and showing highly promising evidence of therapeutic activity. Here we evaluated RRV-mediated prodrug activator gene therapy as a new therapeutic approach for pancreatic ductal adenocarcinoma (PDAC). RRV spread rapidly and conferred significant cytotoxicity with prodrug in a panel of PDAC cells. Efficient intratumoral replication and complete inhibition of tumor growth upon 5-FC administration were observed in both immunodeficient and immunocompetent subcutaneous PDAC models. Biodistribution of RRV was highly restricted in normal tissues, especially in immunocompetent hosts. Tumor growth inhibition by Toca 511 followed by 5-FC was also confirmed in the orthotopic PDAC model. This study provides the first proof-of-concept for application of Toca 511 and Toca FC (extended release 5-FC) to the treatment of human PDAC, and provided support for inclusion of PDAC in a Phase I study evaluating Toca 511 in various systemic malignancies, (NCT02576665), which has recently been initiated.

Introduction

The incidence and death rates of pancreatic ductal adenocarcinoma (PDAC), one of the most common and lethal cancers, are dramatically increasing [1, 2]. Within the next two decades, total deaths due to PDAC are projected to become the second leading cause of cancer-related death [2]. Less than 20% of PDAC patients present with resectable disease [3, 4], and >50% are diagnosed as metastatic disease [1], for which the median survival period is <1 year despite combination chemotherapy [5, 6]. The remaining cases are diagnosed as locally advanced primarily unresectable disease and treated mainly with chemotherapy alone or chemoradiotherapy; however, the efficacy and safety of standard treatments remain insufficient [7]. Thus, the poor clinical outcomes of patients with PDAC emphasize the need to develop novel therapeutic strategies.

In recent years, clinical trials of gene therapy and oncolytic virotherapy for cancer have demonstrated feasibility and safety [8–11], including early- and advanced-phase clinical trials aimed at the treatment of PDAC [12–16]. Although phase I/II trials do not aim to directly evaluate therapeutic efficacy, early-phase trials for advanced-stage PDAC have fallen short of expectations from preclinical studies [12, 15, 16]. Furthermore, the first phase III trial of TNF gene transfer therapy mediated by a replication-defective adenoviral vector in patients with locally advanced PDAC demonstrated a favorable safety profile, but failed to improve survival [14]. In a clinical setting, high-metastatic potential and rapid progression of PDAC pose significant challenges to overcoming this disease by gene therapy. One highly promising approach currently under evaluation in the clinic is to optimize the efficiency of

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gene delivery by the use of tumor-selectively retroviral replicating vectors (RRVs) to PDAC.

RRVs used in this study have been developed based on murine ecotropic gamma retroviruses substituted with the amphotropic (4070A) envelope gene. RRVs are capable of efficient replication and transduction selectively throughout cancer cells, as they can only infect actively dividing cells and are restricted in normal tissues by innate and adaptive immunity, which are impaired or suppressed in the tumor microenvironment. We have previously demonstrated significantly increased survival in orthotopic glioma models when RRVs were employed for highly efficient tumor-selective delivery of prodrug activator genes [17–20]. The evolving preclinical development of 'Toca 511' (*vocimagene amiretrorepvec*), an optimized RRV encoding yeast cytosine deaminase (yCD), which converts the antifungal prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU), has established the foundation for clinical translation of RRV as a potential novel cancer therapy [21, 22]. In preclinical glioma models, Toca 511 spread by replication and tumor transduction, leading to pvector integration into the cancer cell genome, enabling long-term persistence of intratumoral virus infection, and significant cell killing caused by high intratumoral concentrations of 5-FU generated by infected cancer cells following systemic 5-FC administration [21, 23]. As 5-FU has a very short half-life and is generated locally within the tumor itself, there have been no clinically meaningful adverse effects typically associated with conventional systemic chemotherapy, such as myelotoxicity. Also durable anti-tumor immunity was activated through bystander effects on immunosuppressive stromal cells adjacent to infected cancer cells [24, 25]. In multi-center Phase I dose escalation trials for patients with recurrent high-grade glioma, Toca 511 and Toca FC (an extended release formulation of 5-FC) prodrug activator gene therapy showed no clinical meaningful toxicity and highly promising evidence of clinical activity [22], and is now being evaluated in a registrational Phase II/III clinical trial (NCT02414165). Furthermore, based on evidence for therapeutic activity reported in a variety of experimental cancer models [26–28], a new first-in-human Phase I clinical trial evaluating intravenous delivery of Toca 511 in systemic cancers (brain metastases from lung cancer or breast cancer, colorectal cancer with liver metastases, metastatic renal cell carcinoma, melanoma) was initiated in mid-2016 (NCT02576665).

Recently, 5-FU and tegafur/gimeracil/oteracil potassium (S-1), an orally available combination of a 5-FU precursor (tegafur), an inhibitor of 5-FU degradation (gimeracil) and a gut-localized inhibitor of tegafur activation (oteracil), has been shown to be active for PDAC [5, 29–32], and is approved in Japan, but not, so far, in the USA. These agents eventually exert cytotoxic effects by two mechanisms: inhibition of protein synthesis by incorporation into RNA and interference with DNA synthesis by inhibition of thymidylate synthetase [33]. A series of studies evaluating the effect of fluorouracil-based regimens on PDAC show that, in addition to gemcitabine, these are the drugs of choice for treating metastatic or locally advanced unresectable PDAC [5, 31] and also used as adjuvant chemotherapy in patients with resected PDAC [29, 30, 32]. Hence, we hypothesized that Toca 511/5-FC gene therapy, which generates tumor-localized highly concentrated 5-FU, may have more therapeutic effect in PDAC.

Finally, we have recently shown in animal models that, as well as killing cancer cells, Toca 511 and 5-FC treatment depletes local myeloid derived suppressor cells (MDSC) in infected tumors, leading to robust anti-tumor immune responses [24, 25]. Pancreatic cancer is known to carry a significant population of MDSC both in tumors and the blood stream [34]. The Pan02 mouse model used here also shows high levels of MDSC in the tumor [35].

Accordingly, in the current study, we evaluated RRV replication kinetics, tumor transduction efficiency, systemic biodistribution, and therapeutic activity of RRV-mediated prodrug activator gene therapy in preclinical PDAC animal models. Our findings indicate that RRV can achieve efficient replication in human and murine pancreatic cancer cells and efficient intratumoral spread in PDAC models, and that Toca 511-mediated prodrug activator therapy strongly inhibited PDAC tumor growth in both immunodeficient and immunocompetent models.

Materials and methods

Cell lines and culture

Human PDAC cell lines (MIAPaCa-2, BxPC-3) and 293T human embryonic kidney cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). PANC-1 and SUIT-2 were purchased from RIKEN (Tokyo, Japan). Murine PDAC cell line Pan02 was obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute (Frederick, MD). All cell lines were cultured in appropriate medium (MIAPaCa-2, PANC-1, Pan02 and 293T in Dulbecco's Modified Eagle's Medium, BxPC-3 in Roswell Park Memorial Institute Medium, SUIT-2 in Eagle's Minimum Essential Medium, all media from WAKO, Tokyo, Japan) containing 10% fetal bovine serum (Cell Culture Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin (Life Technologies, Tokyo, Japan) at 37 °C with 5% CO_2.

Plasmid constructs and RRV production

The plasmids pAC3-GFP and pAC3-yCD2, which encode Moloney murine leukemia virus with an amphotropic

envelope (4070A) and carrying an internal ribosome entry site (IRES)-transgene (emerald GFP or modified yCD2, respectively) cassette immediately downstream of the envelope stop codon, have been described previously [21]. RRVs were produced by 293T cell transfection with pAC3-GFP or pAC3-yCD2 using Fugene HD Transfection Reagent (Promega, Madison, WI) per manufacturer's instructions, and conditioned medium containing RRV (RRV-GFP or Toca 511, respectively) was collected and filtered through a 0.45 μM filter. Concentrated RRV preparations produced and formulated as described [23] were provided by Tocagen Inc.

Replication kinetics of RRV in PDAC cell lines

Human and murine PDAC cell lines were initially infected with RRV-GFP at a multiplicity of infection (MOI) of 0.05 (~5% initial transduction levels) on day 0. On day 3, one-third of the cells were passaged, and GFP expression was measured by flow cytometry using a FACSCanto II (BD Biosciences, San Jose, CA) running FlowJo software V7.6.5 (Tree Star Inc., Ashland, OR). These steps were repeated every 3 days for human PDAC cell lines or 2 days for the murine PDAC cell line. For MIAPaCa-2 and Pan02 cells, the remaining cells from each passage were washed with PBS, and the cell suspension stored frozen for genomic DNA extraction and further analysis by quantitative real-time PCR (qPCR). In control cultures, a reverse transcriptase inhibitor, 3′-azide-3′deoxythymidine (AZT; Sigma-Aldrich, St. Louis, MO), was added to the medium on day 3 at concentrations of 25–50 μM to prevent viral replication.

qPCR analysis of RRV replication kinetics in vitro

Genomic DNA (gDNA) was extracted from MIAPaCa-2 and Pan02 cell pellets using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) for detection of integrated RRV by qPCR performed in duplicate on a CFX96 Touch System (Bio Rad, Hercules, CA) using TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Waltham, MA) and specific primers and probes, which were designed to target the 4070A amphotropic *env* gene (4070A-F, 5′-GCGGACCCGGACTTTTGA-3′; 4070A-R, 5′-ACCCCGACTTTACGGTATGC-3′; probe, FAM-CAGGGCACACGTAAAA-NFQ). Human RNase P (hRNase P, TaqMan Copy Number Reference Assay, Applied Biosystems) or mouse β-actin (mβ-actin) were quantified as internal control genes (β-actin-F, 5′-GGTCG-TACCACAGGCATTGT-3′; β-actin-R, 5′-CTCGTAGAT-GGGCACAGTGT-3′; probe, FAM-CCCGTCTCCGGA-GTCC-NFQ). A reference curve for RRV copy number was generated with serial dilutions of pAC3-yCD2 in a background of gDNA from uninfected cells. The vector copy numbers in MIAPaCa-2 cells were calculated based on their triploid genome.

In vitro cytotoxicity assay

MIAPaCa-2 or Pan02 cells fully pretransduced with RRV-GFP or Toca 511, as confirmed by >90% GFP-positivity by flow cytometry or RRV copy number by genomic qPCR, and non-transduced parental cells, were seeded in triplicate and exposed to various concentrations of 5-FC (Sigma-Aldrich) for 4 days. Cell viability was measured by tetrazolium dye conversion [3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS)] assay (Promega, Madison, WI) and calculating the optical absorbance of viable cells as measured against control wells without 5-FC.

In vivo studies in PDAC models

Mice

Female BALB/c-nu/nu and C57BL/6 mice (6- to 8-week old, CLEA, Tokyo, Japan) were housed under specific pathogen-free conditions. All animal studies were conducted under protocols approved by the Hokkaido University Animal Research Committee.

Replication kinetics of RRV in subcutaneous (s.c.) tumors

Subcutaneous tumors were established by right dorsal flank injection of uninfected (99%) and RRV-GFP-infected (1%) MIAPaCa-2 cell mixtures (5×10^6 cells/90 μL total volume, in 3:1 chilled Matrigel (Corning, Tewksbury, MA): Hanks' Balanced Salt Solution (HBSS, Life technologies, Carlsbad, CA) in nude mice, and similarly, 99% uninfected/1% RRV-GFP-infected Pan02 cell mixtures (3×10^6 cells/90 μL total volume, in chilled HBSS only) implanted into C57BL/6 mice. Tumors were excised and digested in collagenase/dispase (Roche Diagnostics, Mannheim, Germany) on days 7 and 14 post implantation ($n = 3$ each), and disaggregated cell suspensions from MIAPaCa-2 tumors were immediately stained with anti-human HLA-ABC antibody (eBiosciences, San Diego, CA) to distinguish the human cancer cells from normal mouse cells, while Pan02 tumor suspensions were explanted into medium containing 50 μM AZT for 3 days to prevent RRV replication, followed by flow cytometric analysis of GFP expression.

Therapeutic efficacy in s.c. tumor models

Cell suspensions of uninfected parental MIAPaCa-2 or Pan02 cells (99%) mixed with Toca 511-transduced cells

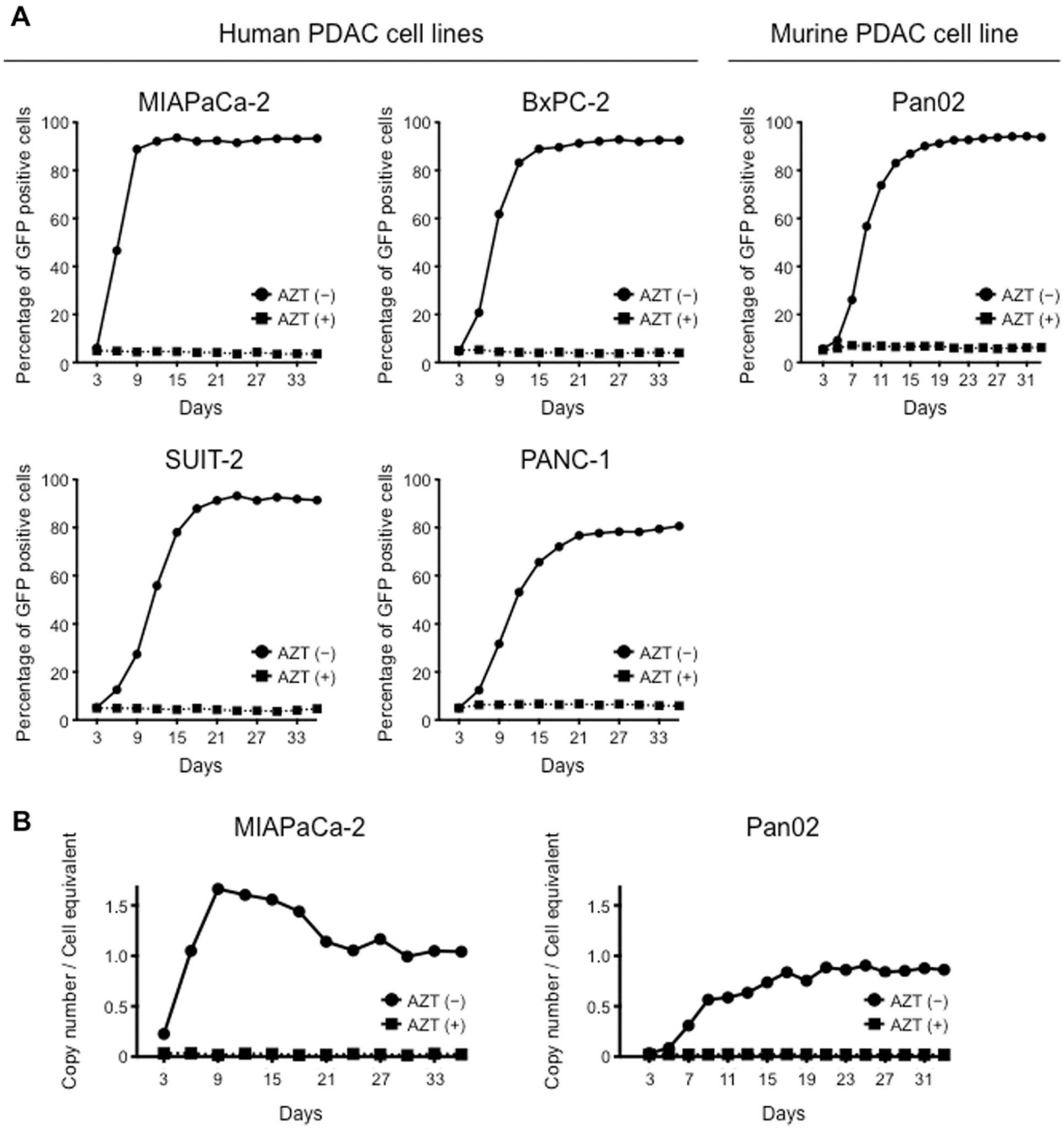

Fig. 1 Replication kinetics of RRV-GFP in PDAC cell lines. **a** Each cell line was initially infected with RRV-GFP at an MOI of 0.05 on day 0, and analyzed for GFP expression by flow cytometry every 3 or 2 days. As a control, a reverse transcriptase inhibitor, AZT was added on day 3 at concentrations of 25 μM (BxPC-3) or 50 μM (other cell lines). **b** in MIAPaCa-2 and Pan02 cell lines, gDNA was extracted from the same cell suspensions as in Fig. 1a and analyzed by qPCR to determine RRV copy number at each time point

(1%) were used to establish s.c. tumors as above. As controls, uninfected parental tumors were also implanted in additional mice. After tumor establishment, mice were randomized to 5-FC treatment groups or PBS control groups ($n = 8$ per group). For 5-FC treatment groups, intraperitoneal (i.p.) 5-FC administration (500 mg/kg once per day) was started 15 days after tumor cell inoculation. For control groups, PBS was administered instead of 5-FC. Daily i.p. administration of 5-FC or PBS was continued up to 60 days in immunodeficient models or 45 days in immunocompetent models, respectively. Tumor volumes were calculated by this formula: volume = length × width^2/2.

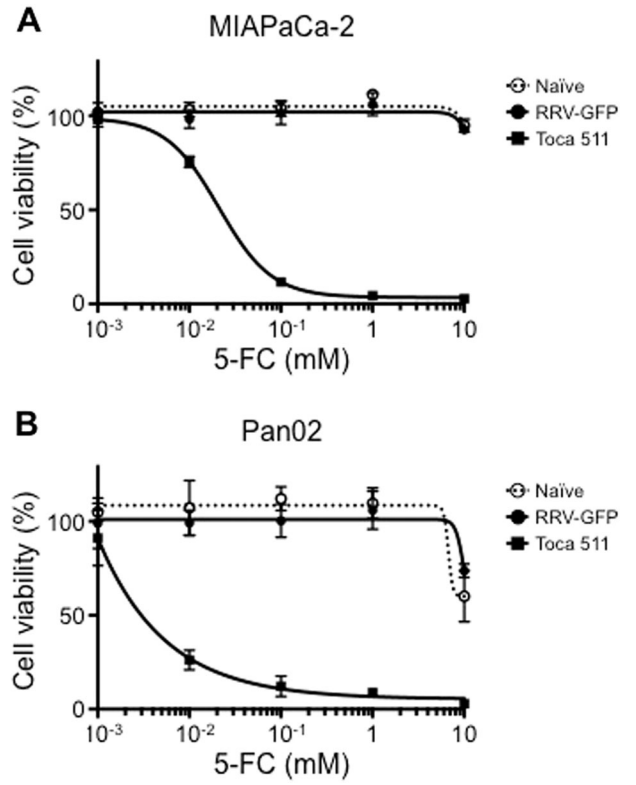

Fig. 2 Prodrug-induced cytotoxicity in Toca 511-transduced PDAC cell lines. Human PDAC cell line, MIAPaCa-2 (**a**) and mouse PDAC cell line, Pan02 (**b**) were cultured for 4 days in a series of 5-FC concentrations. Cell viability was assessed using MTS assay. Data are shown as the percentage (mean ± SD) of viable cells from triplicate wells. Naive: uninfected cells, RRV-GFP: fully RRV-GFP-transduced cells, Toca 511: fully Toca 511-transduced cells

Analysis of RRV biodistribution

Tissues (heart, lung, esophagus, liver, kidney, ovary, rectum, pancreas, spleen, and bone marrow) were collected from mice treated with Toca 511/5-FC ($n = 3$), and gDNA extracted from each tissue using the DNeasy Blood & Tissue Kit (QIAGEN) for qPCR as above, using the mouse β-actin internal control reference curve. The threshold for RRV detection was determined from untransduced control samples.

Replicative spread and therapeutic efficacy of RRV in orthotopic pancreatic cancer models

MIAPaCa-2 cells were transduced with a replication-defective lentivirus vector expressing firefly luciferase, pLenti CMV V5-Luc blast (Addgene, Cambridge, MA). These cells (MIAPaCa-2.Fluc) were tested for luciferase expression by IVIS Spectrum in vivo imaging system (Xenogen/Perkin-Elmer, Waltham, MA); after plating serial dilutions of MIAPaCa-2.Fluc cells, bioluminescent signals were analyzed 15 min after addition of 150 μg/well D-luciferin (OZ Biosciences, San Diego, CA) with a 30-s acquisition time.

For analysis of replicative spread of RRV in orthotopic pancreatic cancer models, uninfected parental MIAPaCa-2. Fluc cells (99%) were mixed with RRV-GFP-transduced MIAPaCa-2.Fluc cells (1%), and 5×10^5 cells were suspended in a 3:1 mixture of ice-cold Matrigel: HBSS (total volume 30 μL). Nude mice were anesthetized with an i.p. injection of ketamine (100 mg/kg; Fujita Pharmaceutical, Tokyo, Japan) and xylazine (10 mg/kg; Sigma-Aldrich), and subcapsular injection of the mixed cell suspension into the pancreatic tail was performed through a left subcostal incision. Fourteen days after tumor cell inoculation, pancreatic tumors were collected, digested, and analyzed by flow cytometry as described above ($n = 3$).

To assess therapeutic efficacy of Toca 511/5-FC treatment in the orthotopic model, pancreatic tumors were established as above using Toca 511-transduced cells instead of RRV-GFP-transduced cells. Each week, 15 min after i.p. administration with D-luciferin (150 mg/kg), bioluminescent signals were analyzed in anesthesized mice by IVIS optical imaging system with a 30-s acquisition time, as above, to monitor tumor growth. After tumor establishment, mice were randomized to 5-FC-treated or PBS control groups ($n = 7$ for each group). For the 5-FC treatment group, 5-FC administration (500 mg/kg i.p., once per day) was started 14 days after tumor cell inoculation. For control groups, PBS was administered daily instead of 5-FC for 5 weeks. The data were analyzed using Living Image Ver.4.2 (Caliper, Hopkinton, MA). Each mouse was also weighed every week for the duration of the experiment. Mice treated with 5-FC were also analyzed for systemic RRV biodistribution ($n = 3$).

Statistical analyses

Statistical analyses were performed with GraphPad Prism 7 software (GraphPad, La Jolla, CA) to determine significance at p-values of <0.05 by Student's t-test. Correlation coefficient (r^2) values >0.9 were defined as a strong correlation. The values are presented as mean ± standard deviation (SD).

Results

RRV shows rapid replication and efficient transduction in PDAC cell lines

To assess RRV replication kinetics in vitro in PDAC cells, we analyzed the percentage of GFP-positive cells over time by flow cytometry and RRV copy number by

quantitative PCR after infection with RRV-GFP. In all human pancreatic cancer cell lines, RRV-GFP inoculated at MOI = 0.05 showed rapid viral replication as indicated by increasing percentages of GFP-positive cells over time, with the majority of pancreatic cancer lines (MIAPaCa-2, BxPC-3, SUIT-2) subsequently reaching >90% transduction (Fig. 1a). MIAPaCa-2 in particular showed robust viral replication and reached >90% transduction by day 9. In murine Pan02 pancreatic cancer cells, the percentage of GFP-expressing cells also increased rapidly, and reached ~90% within 2 weeks (Fig. 1a). In the presence of AZT, viral replication was completely prevented, as indicated by stable percentages of ~5% GFP-positive cells without further increase over time. PCR quantitation of genomic RRV copy number also confirmed efficient transduction by RRV in both MIAPaCa-2 and Pan02 cells (Fig. 1b).

Toca 511 transduction mediates significant cytotoxicity to PDAC cells upon prodrug treatment in vitro

To investigate the cytotoxicity induced by Toca 511/5-FC in PDAC cells in vitro, we measured cell viability at increasing concentrations of 5-FC by MTS assay. In Toca 511-transduced MIAPaCa-2 cells, 88.3 ± 0.8% and 95.6 ± 0.2% reductions in cell viability were induced by exposure to 0.1 mM and 1 mM 5-FC prodrug for 4 days, respectively, as compared to untransduced and RRV-GFP-transduced controls which showed no significant cytotoxicity (Fig. 2a). In Toca 511-transduced Pan02 cells, viability was reduced by 73.8 ± 5.2% after 4-day exposure to only 0.01 mM of 5-FC, and 87.8 ± 5.4% and 91.2 ± 1.5% reductions in cell viability was observed at 0.1 mM and 1 mM 5-FC concentrations, respectively (Fig. 2b).

RRV shows efficient intratumoral spread in s.c. PDAC tumor models

The efficiency of intratumoral RRV spread in PDAC s.c. tumors implanted with 1% RRV-GFP-transduced cells was assessed by flow cytometry. In MIAPaCa-2 xenograft models implanted in immunodeficient hosts, on day 7 post inoculation, 63.9 ± 3.9% of the disaggregated tumor cells stained with human-specific anti-HLA-ABC antibody were positive for GFP expression, and on day 14, the percentage of GFP positive cells had increased to 72.2 ± 9.3% (Fig. 3a, Supplementary Fig. S1A). Similarly, in Pan02 syngeneic models in immunocompetent hosts, the percentage of GFP-positive cells was 61.0 ± 4.9% on day 7, and 72.9 ± 3.9% on day 14, respectively (Fig. 3b, Supplementary Fig. S1B).

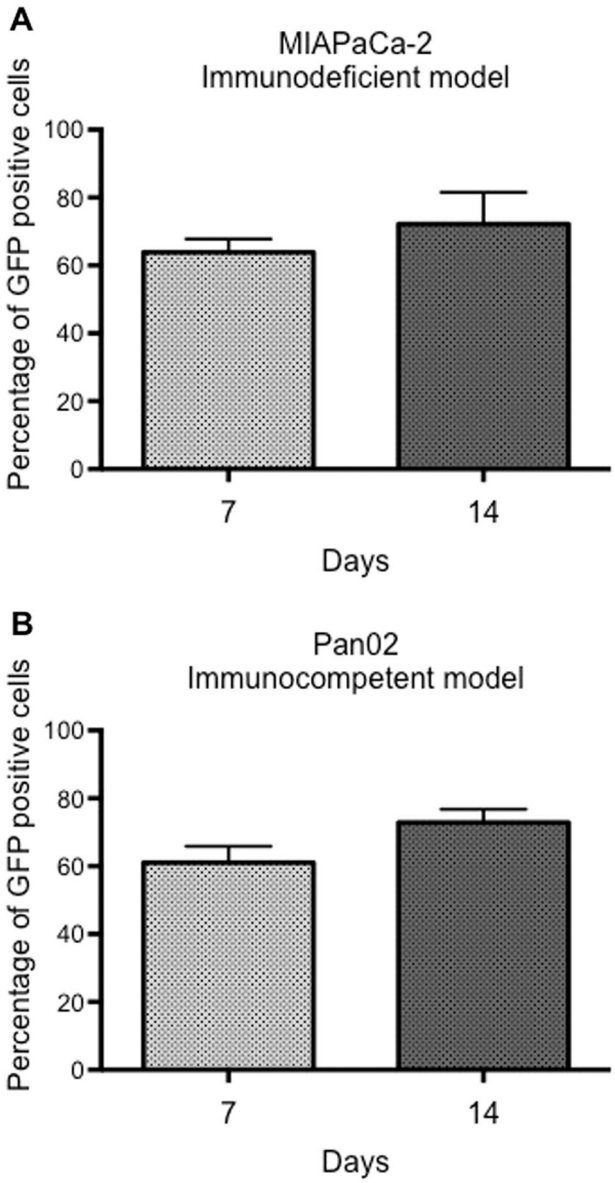

Fig. 3 Intratumoral replicative spread of RRV-GFP in PDAC s.c. tumors. Tumor cells containing 1% fully RRV-GFP-transduced cells were implanted subcutaneously in each mouse. Mice were killed on day 7 or 14 post tumor inoculation, and tumors were excised and enzymatically digested. **a** MIAPaCa-2 immunodeficient mouse models. Tumor cells were immediately stained with anti-HLA-ABC and analyzed for GFP fluorescence by flow cytometry. Percentages of GFP$^+$ HLA-ABC$^+$ cells were quantified. **b** Pan02 immunocompetent mouse models. Tumor cells were cultured in medium containing 50 μM AZT, and after 3-day culture, analyzed for GFP positivity by flow cytometry. $N = 3$ for each group. Data are shown as the mean ± SD

Toca 511/5-FC gene therapy achieves significant antitumor effects in s.c. tumor models

We evaluated in vivo therapeutic efficacy of Toca 511/5-FC treatment using PDAC s.c. tumors implanted with 0% (untransduced) or 1% Toca 511-transduced cells. In both

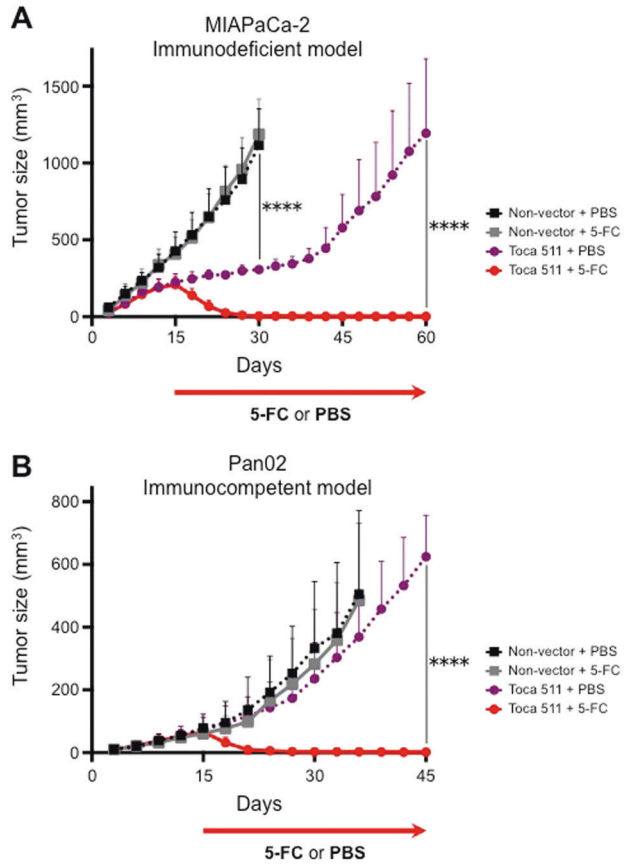

Fig. 4 Therapeutic activity of Toca 511 and 5-FC therapy in PDAC s.c. tumor models. Uninfected cells (99%) were mixed with fully Toca 511-transduced cells (1%), and these cell mixtures or untransduced control cells (100%) were implanted subcutaneously in each mouse. 5-FC (500 mg/kg/day) or PBS i.p. administration was commenced 15 days after tumor cell inoculation. **a** MIAPaCa-2 immunodeficient mouse models. 5-FC or PBS administration was continued up to 60 days. **b** Pan02 immunocompetent mouse models. 5-FC or PBS administration was continued up to 45 days. $N = 8$ for each group. Data are shown as the mean ± SD. Statistical analyses were performed with Student's *t*-test (****$p < 0.0001$)

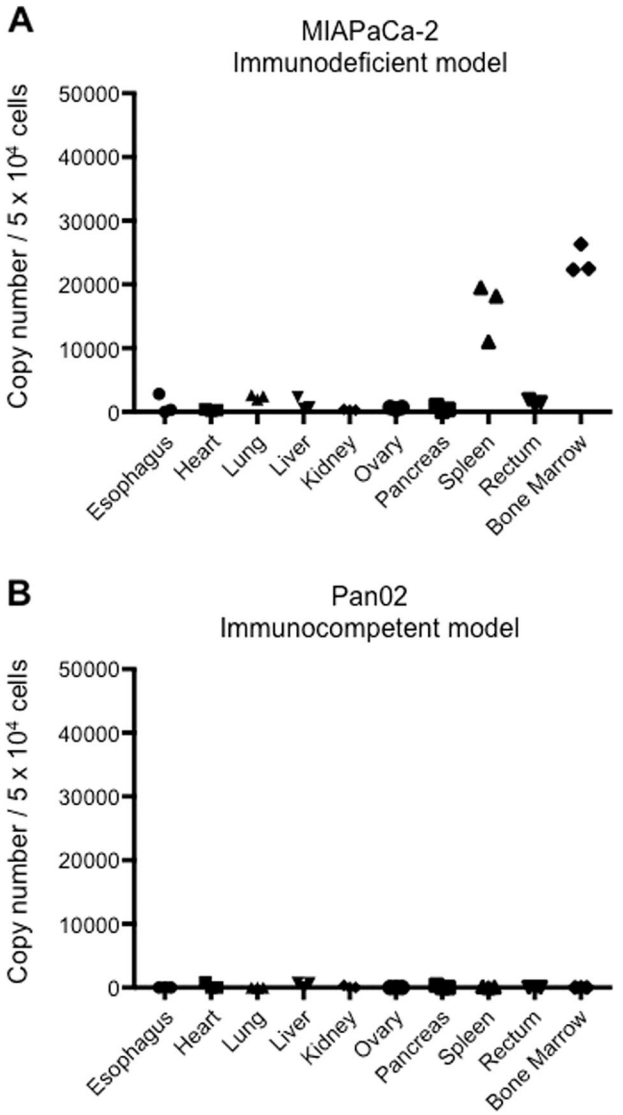

Fig. 5 Analysis of RRV biodistribution in PDAC s.c. tumor models. gDNA extracted from tissues of treated mice in Fig. 4 were analyzed for RRV copy number by qPCR. RRV copy number in different organs in immunodeficient (**a**) and immunocompetent (**b**) mouse models. $N = 3$ for each organ

MIAPaCa-2 and Pan02 s.c. tumor models, controls receiving 5-FC treatment without virus transduction (non-vector + 5-FC group; Fig. 4a, b) showed no obvious inhibition of tumor growth, similar to the completely untreated control group (non-vector + PBS group; Fig. 4a, b). On the other hand, growth of Toca 511-transduced tumors was significantly inhibited by 5-FC treatment, and complete tumor regression was observed in both models ($n = 7/8$) (Toca 511 + 5-FC group; Fig. 4a, b). Interestingly, in the MIAPaCa-2 model, there was about a month delay in the growth of tumors transduced with Toca 511, even without 5-FC treatment (Toca 511 + PBS group; Fig. 4a) compared to the non-treated control tumors. In the Pan02 model, growth delays of Toca 511-transduced tumors without 5-FC (Toca 511 + PBS group; Fig. 4b) was also observed, but was less than that in MIAPaCa-2 model.

Minimal systemic biodistribution after Toca 511/5-FC in immunocompetent PDAC s.c. tumor models

Systemic biodistribution of RRV in mice showing complete tumor regression after treatment with Toca 511/5-FC was analyzed by qPCR of integrated RRV copies in genomic DNA from normal tissues. In immunodeficient mouse models, non-lymphohematopoietic tissues showed only low levels of RRV signals, although there were relatively high levels observed in spleen and bone marrow (Fig. 5a). By contrast, in immunocompetent mouse models, RRV copies were at almost undetectable levels in all tissues tested, showing significant tumor selectivity (Fig. 5b).

RRVs replicate efficiently and achieve significant tumor growth inhibition upon prodrug treatment in orthotopic human pancreatic cancer models

Stable expression of firefly luciferase in MIAPaCa-2.Fluc cells was confirmed by optical imaging, which showed a proportionate relationship between cell number and luminescent signal intensity (Supplementary Fig. S2).

RRV replication was then tested in orthotopic PDAC models initially implanted with 1% RRV-GFP-transduced cells. Similar to the results obtained in s.c. models, RRV again showed efficient intratumoral spread in MIAPaCa-2.FLuc orthotopic models, with 60.9 ± 4.0% of tumor cells, identified by staining with fluorescent APC anti-human HLA-ABC antibody, showing positive GFP expression on day 14 post inoculation (Fig. 6a).

We next assessed the therapeutic efficacy of Toca 511/5-FC therapy by optical imaging in orthotopic MIAPaCa-2.FLuc models, again initially implanted with 1% Toca 511-transduced cells. Untreated control mice showed tumor progression as evidenced by increasing bioluminescence signal intensity, but in Toca 511/5-FC-treated mice, significant inhibition of bioluminescent signals was observed after 5-FC administration (Fig. 6b, c). As expected, marked tumor regression was also observed in the Toca 511/5-FC treatment group, as compared to the untreated control group, by macroscopic pathology findings (Supplementary Fig. S3A), and tumor weights were significantly lower in the treatment group than the control group (Supplementary Fig. S3B). On the other hand, body weight changes were similar in both groups (Supplementary Fig. S3C). Further, systemic biodistribution analysis showed that, as previously, RRV signals were mainly restricted to lymphohematopoietic tissues in this immunodeficient xenograft model (Supplementary Fig. S3D).

Discussion

This is the first preclinical study demonstrating that RRV-mediated prodrug activator gene therapy shows promise as a novel therapy against PDAC. Here we have shown that [1] RRV achieves rapid spread and stable integration in PDAC cells both in vitro and in vivo [2], RRV Toca 511 efficiently delivers the yCD prodrug activator gene to PDAC cells, resulting in significant cancer cell-selective cytotoxicity in vitro and anti-tumor efficacy in vivo upon 5-FC prodrug treatment, and [3] RRV copy numbers in normal tissues are low, particularly in immunocompetent hosts.

As shown previously in a variety of other malignancies [27, 28, 36], here we show that RRV also shows efficient and selective infection of all human and murine PDAC cells tested in vitro. After inoculation of RRV-GFP, the percentage of GFP positive cells increased logarithmically and remained persistently high, at levels of 90% or more, through serial passages. Quantitative PCR analysis of genomic DNA from RRV-infected human and murine PDAC cells showed that the vector copy number also remained stable over serial passage. These results indicate that RRV is capable of non-cytolytic efficient replication and permanent genomic integration in all PDAC cells tested, and each transduced tumor cell itself becomes a stable RRV producer cell, thereby achieving efficient horizontal transmission and expression of the transgene in vitro.

On the other hand, in vivo viral infection and propagation generally shows different kinetics from those in cultured cells. Antiviral innate and adaptive immune responses have been recognized as primary impediments to viral infection and replication [11, 37], yet tumors frequently show defects in innate immunity and suppression of adaptive immunity. Therefore, to obtain more reliable preclinical results for clinical translation, we performed studies in both immunocompetent and immunodeficient models. Since, RRVs can infect both human and murine cells with similar efficiency, this makes it possible to evaluate viral replication kinetics in immunocompetent syngeneic mouse tumor models, which also showed efficient intratumoral viral spread comparable to that in immunodeficient models yet selectivity to tumors.

Significant cytotoxicity was observed in vitro in Toca 511-transduced PDAC cells when cultured in media containing 0.1 mM 5-FC, which is easily achievable in vivo in a clinical setting without associated toxicity [33]. Moreover, although, the percentages of RRV-infected cells at the initiation of prodrug treatment were estimated to be about 70% in subcutaneous models and 60% in orthotopic models, respectively, Toca 511 and 5-FC gene therapy led to higher anti-tumor activity than might be expected based on the transduction level in both models, suggesting the contribution of a "bystander effect" from intratumorally generated 5-FU [38, 39].

In the current studies, 5-FC was administered daily on a continuous schedule. However, continuous 5-FC dosing may inhibit further virus replication within tumors, and may eventually eliminate any reservoir of RRV-infected tumor cells. In previous studies using different cancer models, it has been found that cyclic dosing of 5-FC may achieve an improved therapeutic effect, especially at lower tumor transduction levels [23, 24, 40]. During 'rest' intervals between 5-FC cycles, RRV may more efficiently resume spread from residual reservoirs of stably infected tumor cells, resulting in re-infection of tumors even as they recur, and thereby enabling multiple cycles of 5-FC to achieve continued therapeutic efficacy even after only a single injection of virus.

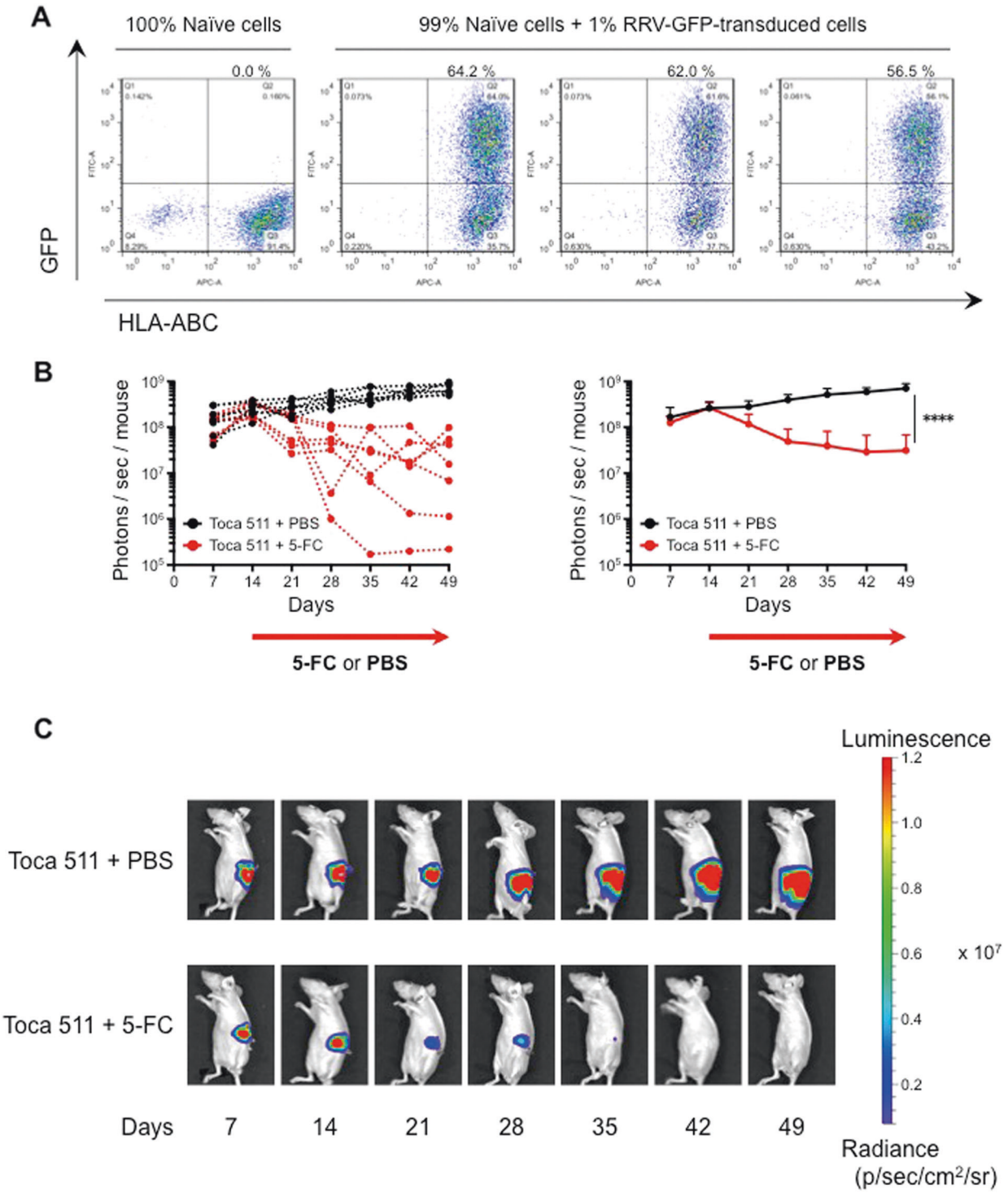

Fig. 6 Replicative spread and therapeutics activity of RRV in orthotopic human pancreatic cancer models. **a** Flow cytometric data demonstrating intratumoral replicative spread of RRV-GFP in orthotopic tumors. MIAPaCa-2.Fluc cells containing 1% fully RRV-GFP-transduced cells were implanted orthotopically in each mouse ($n = 3$). On day 14 post tumor inoculation, tumors were excised and enzymatically digested, stained with anti-HLA-ABC and analyzed for the percentage of GFP positive cells by flow cytometry. Data from a non-vector tumor is shown as a negative control (left). **b** Bioluminescent signal intensities shown as photons/sec/mouse ($n = 7$). MIAPaCa-2.Fluc cells containing 1% fully Toca 511-transduced cells were implanted orthotopically in each mouse. 5-FC (500 mg/kg/day) or PBS administration was commenced on day 14 post tumor inoculation and continued for 5 weeks. Each mouse was analyzed by using IVIS optical imaging system every week. (left) individuals, (right) means. Data are shown as the mean ± SD. Statistical analyses were performed with Student's *t*-test (****$p < 0.0001$). **c** bioluminescent imaging of pancreatic tumors. Representative images for each group are shown

It is also interesting that there was a growth delay in Toca 511-transduced tumors even without 5-FC treatment, which was more pronounced in the MIAPaCa-2 model than in the Pan02 model. Notably, a similar phenomenon was observed in U87 human glioma xenograft models [21]. Although, the precise mechanism of this phenomenon remains unclear, it is possibly related to extra metabolic consumption associated with viral infection and/or production, or alternatively to downregulation of the envelope gene, resulting in reduced phosphate entry [41]. The phenomenon has not been further investigated here.

Systemic biodistribution of RRV was also assessed in both immunodeficient and immunocompetent PDAC models. Both innate host factors, such as APOBEC3 [42] and adaptive immunity contribute to clearance of retroviruses in various species [43–45]. In fact, according to previous reports, amphotropic MLV injected intravenously in normal rhesus monkeys was rapidly cleared from serum without causing subsequent viremia, and no clinical illness was observed in these animals during a mean follow-up period of 43.9 months [46, 47]. Thus, retroviral replication is restricted in normal tissues of immunocompetent hosts. In our immunodeficient mice, RRV vector copy numbers were relatively low or undetectable in most normal tissues in the time-frame examined, except for lymphoid and hematopoietic tissues (spleen, bone marrow), as previously reported [48]. In contrast, in immunocompetent C57BL/6 mice, systemic biodistribution of RRV was significantly restricted in all organs, including lymphohematopoietic tissues. This difference may be due to strain-dependent differences in host restriction factors and/or the immune competence of the host [49]. Importantly, MLV has been shown to be more restricted in humans partly by the effect of APOBEC3G [50], and consequently, minimal RRV infection of normal tissues is expected in humans with normal immunity. Additionally, no symptoms or weight loss were observed during the treatment period, indicating that there is no acute toxicity associated with tumor-selective RRV infection. In addition, Toca 511 and 5-FC mediated prodrug conversion is likely to eliminate inadvertently infected normal dividing cells upon 5-FC administration, thereby acting as an inherent 'self-destruct' mechanism. Hence, the potential risk of genotoxicity through insertional mutagenesis seems very low. Thus, the safety profile of this strategy is expected to be acceptable in treating PDAC, as has been the case in multiple clinical trials using Toca 511 and 5-FC for the treatment of recurrent high-grade glioma.

In conclusion, we show that Toca 511 and 5-FC therapy has good potential for clinical translation and may be a promising therapeutic strategy in PDAC. Currently, this strategy is being evaluated in an exploratory Phase I clinical trial of Toca 511 and Toca FC for the treatment of PDAC and other malignancies.

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Compliance with ethical standards

Conflict of interest D.J.J., and H.E.G. are employees of Tocagen. N.K. is a consultant. All three have an ownership interest in Tocagen. All other authors declare that they have no conflict of interest.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016;66:7–30.
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014;74:2913–21.
3. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. N Engl J Med. 2014;371:1039–49.
4. Werner J, Combs SE, Springfeld C, Hartwig W, Hackert T, Buchler MW. Advanced-stage pancreatic cancer: therapy options. Nat Rev Clin Oncol. 2013;10:323–33.
5. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011;364:1817–25.
6. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013;369:1691–703.
7. Chen Y, Sun XJ, Jiang TH, Mao AW. Combined radiochemotherapy in patients with locally advanced pancreatic cancer: a meta-analysis. World J Gastroenterol. 2013;19:7461–71.
8. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012—an update. J Gene Med. 2013;15:65–77.
9. Brenner MK, Gottschalk S, Leen AM, Vera JF. Is cancer gene therapy an empty suit? Lancet Oncol. 2013;14:e447–56.
10. Singh HM, Ungerechts G, Tsimberidou AM. Gene and cell therapy for pancreatic cancer. Expert Opin Biol Ther. 2015; https://doi.org/10.1517/14712598.2015.1001734
11. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nat Biotechnol. 2012;30:658–70.
12. Hecht JR, Bedford R, Abbruzzese JL, Lahoti S, Reid TR, Soetikno RM, et al. A phase I/II trial of intratumoral endoscopic ultrasound injection of ONYX-015 with intravenous gemcitabine in unresectable pancreatic carcinoma. Clin Cancer Res. 2003;9:555–61.
13. Hecht JR, Farrell JJ, Senzer N, Nemunaitis J, Rosemurgy A, Chung T, et al. EUS or percutaneously guided intratumoral TNFerade biologic with 5-fluorouracil and radiotherapy for first-line treatment of locally advanced pancreatic cancer: a phase I/II study. Gastrointest Endosc. 2012;75:332–8.
14. Herman JM, Wild AT, Wang H, Tran PT, Chang KJ, Taylor GE, et al. Randomized phase III multi-institutional study of TNFerade biologic with fluorouracil and radiotherapy for locally advanced pancreatic cancer: final results. J Clin Oncol. 2013;31:886–94.

15. Nakao A, Kasuya H, Sahin TT, Nomura N, Kanzaki A, Misawa M, et al. A phase I dose-escalation clinical trial of intraoperative direct intratumoral injection of HF10 oncolytic virus in non-resectable patients with advanced pancreatic cancer. Cancer Gene Ther. 2011;18:167–75.
16. Noonan AM, Farren MR, Geyer SM, Huang Y, Tahiri S, Ahn D, et al. Randomized phase 2 trial of the oncolytic virus pelareorep (reolysin) in upfront treatment of metastatic pancreatic adenocarcinoma. Mol Ther. 2016;https://doi.org/10.1038/mt.2016.66
17. Wang WJ, Tai CK, Kasahara N, Chen TC. Highly efficient and tumor-restricted gene transfer to malignant gliomas by replication-competent retroviral vectors. Hum Gene Ther. 2003;14:117–27.
18. Tai CK, Wang WJ, Chen TC, Kasahara N. Single-shot, multicycle suicide gene therapy by replication-competent retrovirus vectors achieves long-term survival benefit in experimental glioma. Mol Ther. 2005;12:842–51.
19. Wang W, Tai CK, Kershaw AD, Solly SK, Klatzmann D, Kasahara N, et al. Use of replication-competent retroviral vectors in an immunocompetent intracranial glioma model. Neurosurg Focus. 2006;20:E25.
20. Tai CK, Wang W, Lai YH, Logg CR, Parker WB, Li YF, et al. Enhanced efficiency of prodrug activation therapy by tumor-selective replicating retrovirus vectors armed with the Escherichia coli purine nucleoside phosphorylase gene. Cancer Gene Ther. 2010;17:614–23.
21. Perez OD, Logg CR, Hiraoka K, Diago O, Burnett R, Inagaki A, et al. Design and selection of Toca 511 for clinical use: modified retroviral replicating vector with improved stability and gene expression. Mol Ther. 2012;20:1689–98.
22. Cloughesy TF, Landolfi J, Hogan DJ, Bloomfield S, Carter B, Chen CC, et al. Phase 1 trial of vocimagene amiretrorepvec and 5-fluorocytosine for recurrent high-grade glioma. Sci Transl Med. 2016;8:341ra75.
23. Ostertag D, Amundson KK, Lopez Espinoza F, Martin B, Buckley T, Galvao da Silva AP, et al. Brain tumor eradication and prolonged survival from intratumoral conversion of 5-fluorocytosine to 5-fluorouracil using a nonlytic retroviral replicating vector. Neuro Oncol. 2012;14:145–59.
24. Hiraoka K, Inagaki A, Kato Y, Huang TT, Mitchell LA, Kamijima S, et al. Retroviral replicating vector-mediated gene therapy achieves long-term control of tumor recurrence and leads to durable anticancer immunity. Neuro Oncol. 2017;https://doi.org/10.1093/neuonc/nox038
25. Mitchell LA, Lopez Espinoza F, Mendoza D, Kato Y, Inagaki A, Hiraoka K, et al. Toca 511 gene transfer and treatment with the prodrug, 5-fluorocytosine, promotes durable antitumor immunity in a mouse glioma model. Neuro Oncol. 2017;https://doi.org/10.1093/neuonc/nox037
26. Hiraoka K, Kimura T, Logg CR, Kasahara N. Tumor-selective gene expression in a hepatic metastasis model after locoregional delivery of a replication-competent retrovirus vector. Clin Cancer Res. 2006;12:7108–16.
27. Hiraoka K, Kimura T, Logg CR, Tai CK, Haga K, Lawson GW, et al. Therapeutic efficacy of replication-competent retrovirus vector-mediated suicide gene therapy in a multifocal colorectal cancer metastasis model. Cancer Res. 2007;67:5345–53.
28. Hickey MJ, Malone CC, Erickson KL, Lin A, Soto H, Ha ET, et al. Combined alloreactive CTL cellular therapy with prodrug activator gene therapy in a model of breast cancer metastatic to the brain. Clin Cancer Res. 2013;19:4137–48.
29. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, et al. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. JAMA. 2010;304:1073–81.
30. Regine WF, Winter KA, Abrams R, Safran H, Hoffman JP, Konski A, et al. Fluorouracil-based chemoradiation with either gemcitabine or fluorouracil chemotherapy after resection of pancreatic adenocarcinoma: 5-year analysis of the U.S. Intergroup/RTOG 9704 phase III trial. Ann Surg Oncol. 2011;18:1319–26.
31. Ueno H, Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, Boku N, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J Clin Oncol. 2013;31:1640–8.
32. Uesaka K, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, et al. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). Lancet. 2016;https://doi.org/10.1016/s0140-6736(16)30583-9
33. Vermes A, Guchelaar HJ, Dankert J. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J Antimicrob Chemother. 2000;46:171–9.
34. Pergamo M, Miller G. Myeloid-derived suppressor cells and their role in pancreatic cancer. Cancer Gene Ther. 2017;24:100–5.
35. Karakhanova S, Link J, Heinrich M, Shevchenko I, Yang Y, Hassenpflug M, et al. Characterization of myeloid leukocytes and soluble mediators in pancreatic cancer: importance of myeloid-derived suppressor cells. Oncoimmunology. 2015;4:e998519.
36. Twitty CG, Diago OR, Hogan DJ, Burrascano C, Ibanez CE, Jolly DJ, et al. Retroviral replicating vectors deliver cytosine deaminase leading to targeted 5-fluorouracil-mediated cytotoxicity in multiple human cancer types. Hum Gene Ther Methods. 2016;27:17–31.
37. Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. Mol Ther. 2007;15:651–9.
38. Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc Natl Acad Sci USA. 1994;91:8302–6.
39. Chen JK, Hu LJ, Wang D, Lamborn KR, Deen DF. Cytosine deaminase/5-fluorocytosine exposure induces bystander and radiosensitization effects in hypoxic glioblastoma cells in vitro. Int J Radiat Oncol Biol Phys. 2007;67:1538–47.
40. Huang T, Parab S, Burnett R, Diago O, Ostertag D, Hofman FM, et al. Intravenous administration of a retroviral replicating vector, Toca 511, demonstrates therapeutic efficacy in an orthotopic immune-competent mouse glioma model. Hum Gene Ther. 2014;https://doi.org/10.1089/hum.2014.100
41. Macdonald C, Walker S, Watts M, Ings S, Linch DC, Devereux S. Effect of changes in expression of the amphotropic retroviral receptor PiT-2 on transduction efficiency and viral titer: implications for gene therapy. Hum Gene Ther. 2000;11:587–95.
42. Takeuchi H, Matano T. Host factors involved in resistance to retroviral infection. Microbiol Immunol. 2008;52:318–25.
43. Takeuchi Y, Cosset FL, Lachmann PJ, Okada H, Weiss RA, Collins MK. Type C retrovirus inactivation by human complement is determined by both the viral genome and the producer cell. J Virol. 1994;68:8001–7.
44. Green WR. Cytotoxic T lymphocytes to endogenous mouse retroviruses and mechanisms of retroviral escape. Immunol Rev. 1999;168:271–86.
45. Williams KC, Burdo TH. HIV and SIV infection: the role of cellular restriction and immune responses in viral replication and pathogenesis. APMIS. 2009;117:400–12.

46. Cornetta K, Moen RC, Culver K, Morgan RA, McLachlin JR, Sturm S, et al. Amphotropic murine leukemia retrovirus is not an acute pathogen for primates. Hum Gene Ther. 1990;1:15–30.
47. Cornetta K, Morgan RA, Anderson WF. Safety issues related to retroviral-mediated gene transfer in humans. Hum Gene Ther. 1991;2:5–14.
48. Lin AH, Timberlake N, Logg CR, Liu Y, Kamijima S, Diago O, et al. MicroRNA 142-3p attenuates spread of replicating retroviral vector in hematopoietic lineage-derived cells while maintaining an antiviral immune response. Hum Gene Ther. 2014;25:759–71.
49. Takeda E, Tsuji-Kawahara S, Sakamoto M, Langlois MA, Neuberger MS, Rada C, et al. Mouse APOBEC3 restricts friend leukemia virus infection and pathogenesis in vivo. J Virol. 2008;82:10998–1008.
50. Browne EP, Littman DR. Species-specific restriction of apobec3-mediated hypermutation. J Virol. 2008;82:1305–13.