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A genetic fiber modification to achieve matrix-metalloprotease-activated infectivity of oncolytic adenovirus

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ABSTRACT

Selective tumor targeting of oncolytic adenovirus at the level of cell entry remains a major challenge to improve efficacy and safety. Matrix metalloproteases (MMPs) are overexpressed in a variety of tumors and in particular in pancreatic cancer. In the current work, we have exploited the expression of MMPs together with the penetration capabilities of a TAT-like peptide to engineer tumor selective adenoviruses. We have generated adenoviruses containing CAR-binding ablated fibers further modified with a C-terminus TAT-like peptide linked to a blocking domain by an MMP-cleavable sequence. This linker resulted in a MMP-dependent cell transduction of the reporter MMP-activatable virus AdTATMMP and in efficient transduction of neoplastic cells and cancer-associated fibroblasts. Intravenous and intraductal administration of AdTATMMP into mice showed very low AdTATMMP activity in the normal pancreas, whereas increased transduction was observed in pancreatic tumors of transgenic Ela-myc mice. Intraductal administration of AdTATMMP into mice bearing orthotopic tumors led to a 25-fold increase in tumor targeting compared to the wild type fiber control. A replication competent adenovirus, Ad^{RC}MMP, with the MMP-activatable fiber showed oncolytic efficacy and increased antitumor activity compared to Adwt in a pancreatic orthotopic model. Reduced local and distant metastases were observed in Ad^{RC}MMP treated-mice. Moreover, no signs of pancreatic toxicity were detected. We conclude that MMP-activatable adenovirus may be beneficial for pancreatic cancer treatment.

1. Introduction

Adenovirus serotype 5 (Ad5) is commonly used in the development of oncolytic agents for cancer gene therapy. Ad5 infectivity depends on the expression of the coxsackievirus–adenovirus-receptor (CAR) on the surface of target cells. However, since CAR is frequently downregulated in tumor cells, Ad5 neoplastic cell transduction is often inefficient, limiting the anti-cancer effects of oncolytic adenoviruses. Hence, efforts to improve adenoviral tumor cell transduction have been developed. Strategies such as fiber pseudotyping [1], the addition of high affinity ligands to different adenovirus capsid proteins [2–5], and the incorporation of chemically or conjugated surface modifications have been studied [6–8]. In particular, the protein transduction domain Tat-PTD from the HIV-1 Tat protein has been inserted into the HI Loop or the C-terminus of the fiber knob [3] and into the hexon hypervariable region 5 (HVR5) of the virus capsid showing increased transduction efficacy on a variety of tumor cells [5].

An optimal retargeted virus should combine an efficient tumor cell transduction with tumor selectivity. Viral activation through cancer-specific proteases is an approach that has been tested to redirect enveloped viruses to cancer cells. Since most enveloped viruses require protease cleavage of viral glycoproteins for productive cell entry, retroviral glycoproteins have been engineered with matrix metalloproteases (MMP)-cleavable linkers to provide retroviruses with cancer specificity [9,10]. Measles and Sendai viruses have also been modified with similar strategies showing tumor selectivity [11,12].

In the current work we studied the potential to confine adenoviral oncolysis to tumor tissue with the dual objective to: i) improve adenoviral tumor transduction and ii) achieve adenoviral specific activation in tumor cells. We have generated a fiber-modified adenovirus with a TAT-like domain exposed upon cleavage with tumor-specific proteases. We show MMP-dependent selectivity of the virus, increased tumor

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targeting and remarkable oncolytic antitumoral efficacy in pancreatic xenografts.

2. Materials and methods

2.1. Cell lines

HEK293, NIH-3T3 cells, and the human pancreatic adenocarcinoma cell line PANC-1 were obtained from the American Type Cancer Collection (ATCC). The human pancreatic adenocarcinoma cell line RWP-1 and the HT1080 fibrosarcoma cell line were kindly provided by Dr F.X. Real and Dr. R. Alemany, respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, and cultured as described [13]. PSC-21, CAF-25, and CAF-28 cells were isolated from fresh pancreatic specimens obtained from adult patients undergoing surgical resection for pancreatic cancer at the Hospital Clinic in Barcelona. All human samples were obtained under the approval of the Institutional Ethics Committee. Tissue blocks were cut into small pieces and dissociated in collagenase solution. Digested tissue was filtered and later centrifuged on a 12% Optiprep density gradient. A resultant fuzzy band at the interface and the cell pellet were collected separately, suspended in DMEM/F-12 medium, and cultured with DMEM-F-12 medium containing 15% FBS, Glutamax, antibiotics, HEPES, insulin, and IGF-1. Fibroblasts collected from the interface were identified as quiescent pancreatic stellate cells (PSCs) and fibroblasts growing out from pellets were considered as cancer associated fibroblasts (CAFs). Activated PSCs and CAFs were characterized by immunostaining. Both cell types showed abundant expression of α-smooth muscle actin and vimentin and were negative for E-cadherin.

2.2. Adenovirus construction

AdCMVGFPLuc (AdTL) has been previously described [14]. Adwt, the wild-type adenovirus, was obtained from the ATCC (Manassas, VA). In the present work we have generated the recombinant adenoviruses AdYTRGE, AdTATMMP, and the replication-competent virus Ad^{RC}MMP. AdTL, AdYTRGE, and AdTATMMP express the enhanced GFP gene and the firefly luciferase gene under the control of a CMV promoter.

The AdYTRGE was generated modifying the plasmid backbone of pAdTL. Three modifications were performed on the fiber gene: i) the Y477A mutation (TAT changed to GCT), ii) deletion of T489 A490 Y491 T492 residues (sequence deleted 5′-ACAGCCTATACA-3′); and iii) insertion of residues SKCDCRGECFCD (sequence inserted: 5′-TCAAAATGTG ACTGCCGCGGAGAATGTTTCTGCGAC-3′) between T546 and P547 of the HI-loop. All modifications were done by homologous recombination in yeast [15].

The AdTATMMP was generated by inserting the TATMMP fragment from the plasmid pGEM-T fiber TATMMP end into the NcoI/MfeI sites of the pXK3.1 plasmid, which contains the wild type fiber, generating the pXK3.1–YTRGE–TATMMP. TATMMP fragment was obtained by cloning TATMMP sequence at the end of the fiber sequence by two consecutive Touch Down-PCR. First, TAT-like (TAT*) sequence was amplified from the plasmid pBSatYTRGE using the primers Fiber1Fw (5′-GCACCAAACACAAATCCC-3′) and FiberRv1 (5′GCGCCGCCTCTTCGTCGC TGTCTCCGCTTCTTGCTGCCATATTCTTGGGCAATGTATGA-3′). The PCR product, that contained the YTRGE mutations and TAT* sequence, was cloned into a T-vector following the manufacturer's instructions (pGEM®-T Easy Vector System I, Promega). Then, the MMP-cleavable linker-Blockage-3′ UTR sequence was amplified from the recently generated plasmid using the primers Fiber1Fw and FiberRv2 (5′-GCAATTGA AAAATAAACACGTTGAAACATAACACAAACGATTCTTTACTCCTCTTCCTCC TCGCCCTCTTCCTCGCCGCCCTTGTACAGGCCCTTGGCGCCGCCTCTTCGTC GCTGTCT-3′) and cloned into a T-vector generating the plasmid pGEM-T fiber TATMMP end. Next, homologous recombination of XbaI/KpnI digested TATMMP fragment with the linearized plasmid pVK50TL (adenoviral genome) was carried following a standard protocol. Viral particles were obtained and propagated in HEK293 cells.

The Ad^{RC}MMP is a replication-competent adenovirus, containing the wild-type Ad5 genome with the modified fiber TATMMP. It was generated by homologous recombination of XbaI/KpnI digested TATMMP fragment with the linearized plasmid pVK50 (adenoviral genome) following standard protocol. Viral particles were obtained in HEK293 cells and propagated in A549 cells.

All viruses were purified by standard cesium chloride banding and the physical particle concentration (vp/ml) was determined by optical density reading (OD_{260}). Viral titers for Adwt: $5{,}61 \times 10^{12}$ vp/ml and for Ad^{RC}MMP: $3{,}79 \times 10^{12}$ vp/ml. Correct fiber was verified by PCR and by PCR-product sequencing using Fiber1Fw, described above, and Fiber4Rv (5′-GTATAAGCTATGTGGTGGTGG-3′) primers.

2.3. Transduction efficiency assays

Cells were seeded in triplicate in 96-well plates. After 24 h, cells were infected with the corresponding adenovirus at 10^3 vp/cell or 10^4 vp/cell. Virus was removed 6 h later and cells were further cultured in a complete medium. Luciferase expression was measured three days later according to manufacturer's instructions (Luciferase Assay System; Promega). For experiments using pre-cleaved AdTATMMP, 24 h after cell seeding, AdTATMMP was incubated without or with different doses of MMP-9 (Calbiochem) in FBS-depleted medium, at 37 °C for 2 h, previous to cell infection.

2.4. In vitro cytotoxicity assays

A total of 3×10^3 cells (HT1080, PANC-1and RWP-1) or 10^3 cells (CAF-28) were seeded in triplicate in a 96-well plate and infected with either Adwt or Ad^{RC}MMP at different doses. Virus was removed 6 h later and cells were further cultured in a complete medium. Cell viability was measured 5 days later by an MTT colorimetric assay (Roche Molecular Biochemicals) (HT1080, PANC-1, and RWP-1) or by methylene blue staining (CAF-28).

2.5. Viral yield

PANC-1 and RWP-1 cells were seeded (4×10^4 cells per well in duplicate in 24-well plates) and infected with either Adwt or Ad^{RC}MMP at 50 vp/cell in the absence of FBS. Virus was removed 6 h later and cells were washed with PBS and cultured in FBS-complete medium. Total cell extracts were collected at 3 and 5 days post-infection and genomic DNA was extracted using UltraClean® BloodSpin® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Viral genomes were determined by Real Time quantitative PCR (qPCR), performed on ViiA™ 7 System (Applied Biosystems, Life Technologies™) using SYBR Green mastermix (Roche) and hexon specific primers: AdHEXO1: GCCGCAGTGGTCTTAC ATGCACATC, AdHEXO2: CAGCACGCCGCGGATGTCAAAG. The adenovirus copy number was quantified with a standard curve, consisting of adenovirus DNA dilutions (10^2–10^7 copies) in a background of genomic DNA. Values were corrected by the number of cells per well, determined by qPCR of albumin intron 4. S-albumin: CTGTCATCTCTTGTGG GCTGT and aS-albumin: GGCTATCCAAACTCATGGGAG.

2.6. MMP expression analysis

Total RNA was obtained from pancreatic tissue with the RNeasy® Mini Kit (Qiagen) and reverse transcribed with Retroscript RT kit (Ambion, Life Technologies ™), using random decamers, in accordance with the manufacturer's protocols. Real Time quantitative PCR was performed on ViiA™ 7 System (Applied Biosystems, Life Technologies ™) using SYBR Green mastermix (Roche) and MMP-2 and GAPDH specific primers: MMP2-Fw: TGATGGCATCGCTCAGATCC, MMP2-Rv: CACAGC

CTTCTCCTCCTGTG, GAPDH-Fw: TGTCAAGCTCATTTCCTGGTATGA, GAPDH-Rv: CTTACTCCTTGGAGGCCATGTAG.

2.7. Mouse models

Animal procedures followed the guidelines of European Community Directive 86/609/EEC and were approved by the Local Ethical Committee. Transgenic Ela-myc and wt C57BL/6 mice of 16 weeks of age were used; they were genotyped by multiplex PCR analysis as previously described [16]. Male athymic nu/nu mice (8 weeks old, Harlan Iberica) were used to generate orthotopic pancreatic tumors. PANC-1 or PANC-1/CAF-25, and PANC-1-Luc/CAF-28 mixed orthotopic tumors were generated by injecting a total of 350,000 cells (ratio 10:1) into the pancreas of athymic nude mice, in a final volume of 50 μl, as previously described [17].

2.8. Bioluminescence assay and quantification of luciferase expression

Firefly D-Luciferin (PerkinElmer, Inc) was administered i.p. (32 mg/kg) to mice and 10 min later were anesthetized with a mixture of isoflurane and oxygen preparation. In vivo and ex vivo luciferase activity was visualized and images were quantified using the Wasabi image analysis software (Hamamatsu Photonics) as described [18].

Luciferase transgene expression in tissue extracts was quantified using the reporter Luciferase Assay System (Promega) as previously described [19].

2.9. Antitumoral efficacy study

PANC-1/CAF-28 tumor-bearing mice were intraductally injected as previously reported [16] with saline, 5×10^{10}vp of Adwt or Ad^{RC}MMP, and tumor growth was monitored by measuring in vivo luciferase emission. Animals were sacrificed 42 days after virus administration and pancreatic-tumor tissue was isolated; metastatic foci were also analyzed. Ex vivo luciferase emission of pancreatic tissue was determined and pancreatic-tumor volume was measured as follows: $V\,(mm^3) = a \times b \times c/2$, where a = length, b = width, and c = height of pancreas.

2.10. Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded tissues were prepared as previously described [17]. Five-micrometer sections were treated with 10 mM citrate buffer (pH 6.0) and incubated overnight at 4 °C with anti-GFP (A6455, Invitrogen) or with anti-α-SMA (ab7817, Abcam) antibodies. Alexa Fluor 488 and Alexa Fluor 633 (A11001 and A21071 respectively, Molecular Probes, Life Technologies™) were used as secondary antibodies. Nucleus were counterstained with 5 μg/ml bis-benzimide (Hoechst 33342; Sigma) and visualized under a fluorescent microscope (Nikon Eclipse 50i). The fluorescent images were captured using a digital camera (Cool Cube1, MetaSystems).

2.11. Toxicity analysis

Blood samples were collected by intracardiac puncture under anesthesia. Serum alanine transaminase (ALT), aspartate transaminase (AST), amylase and lipase were determined on an Olympus AU400 Analyzer.

2.12. Statistical analysis

Results are expressed as mean ± SEM of at least three independent experiments. Statistical differences were determined using Prism (version 5; GraphPad Software Inc.) and were considered significant for P values less than 0.05. A Mann–Whitney nonparametric test was used for the statistical analysis (2-tailed) of in vitro and in vivo studies. Regular two-way ANOVA was used to compare differences between tumor growth curves from saline, Adwt and Ad^{RC}MMP treated groups.

3. Results

3.1. AdTATMMP transduction is activated by matrix metalloproteases MMP2 and MMP9

We hypothesized that an MMP-activatable adenovirus containing a TAT-like peptide in the fiber could selectively enhance tumor cell transduction independently of CAR expression. To test this hypothesis we generated a fiber-modified recombinant adenovirus (AdTATMMP) expressing the reporter gene luciferase and GFP (Fig. 1A and B). Fiber modifications included the insertion of a TAT-derived peptide (TAT*) in the fiber knob domain followed by an MMP-cleavable linker, recognized by MMP-2 and MMP-9, and a polyanionic sequence designed to neutralize the polycations of the TAT sequence by forming intramolecular hairpins (Fig. 1C). To achieve non-tumor cell detargeting, these modifications were engineered in a backbone of a mutated fiber to ablate binding to CAR and to coagulation factor IX (YTRGE mutations) [20]. To first confirm that the MMP-linker was susceptible to be cleaved by matrix metalloproteases in the context of the TAT* peptide and the polyanionic block, a 30-aa peptide containing the indicated sequences was chemically synthesized, incubated in the presence of recombinant MMP-2 protein and analyzed by HPLC at different time points. Cleavage of the peptide was evidenced by the appearance of two new peaks corresponding to the two fragments expected from proteolysis at the Gly – Leu peptide bond of the MMP-linker, and was almost complete at 240 min (Supplementary Fig. S1).

Next, we investigated the capacity of the AdTATMMP to transduce cells in response to protease cleavage. AdTATMMP was incubated with increased concentrations of recombinant MMP-9 protein for 2 h. Then cells were exposed to pre-treated virus and luciferase activity was measured 3 days later. A dose-dependent effect on viral transduction was observed in all the cell lines, with the highest activity observed at the

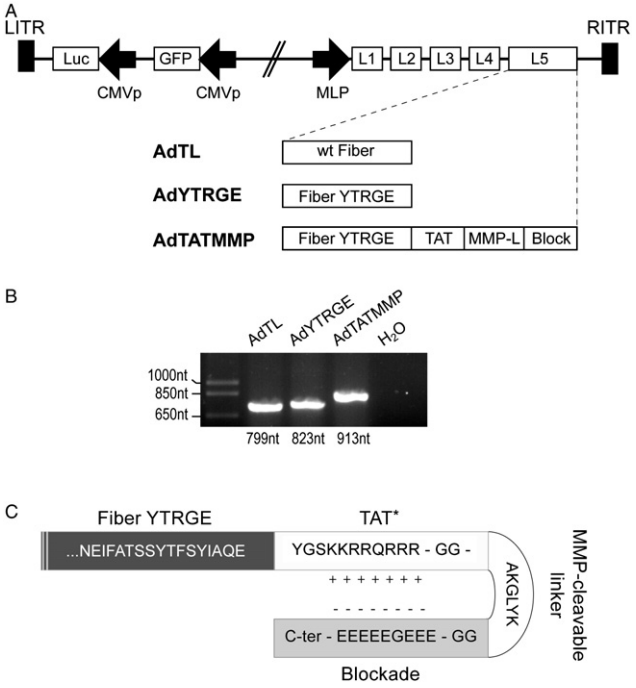

Fig. 1. AdTATMMP adenovirus. A, schematic representation of AdTL, AdYTRGE and AdTATMMP. B, fiber detection: PANC-1 cells were infected with purified adenovirus. Two days later, cells were harvested and total DNA was obtained and used for PCR analyses. C, schematic diagram of C-terminal fiber protein of AdTATMMP. TAT^*_{PTD} is blocked by a stretch of acidic residues attached by an MMP cleavable linker.

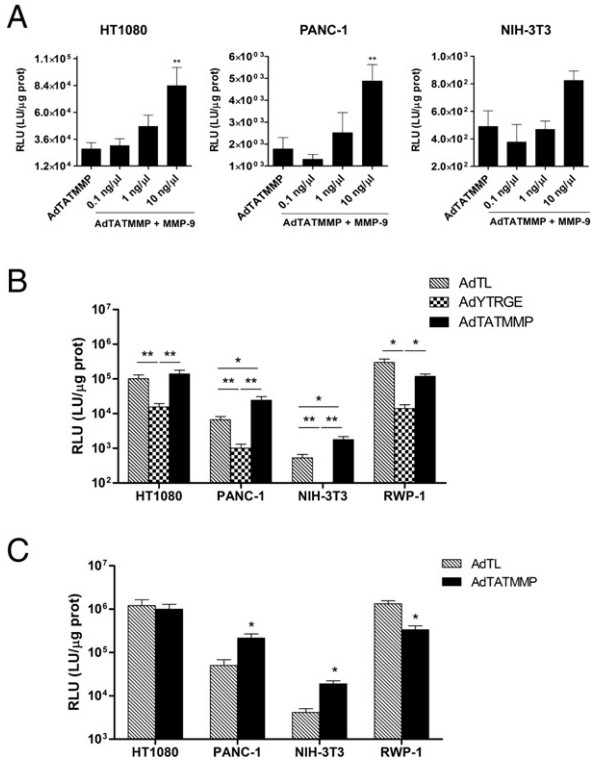

Fig. 2. AdTATMMP transduction efficiency. A total of 10^4 cells were seeded in triplicate and infected with the corresponding adenovirus at 10^3 vp/cell (A, B) and 10^4 vp/cell (C). Luciferase expression was measured three days later. Results are represented as the mean ± SEM of at least 3 independent experiments. A, AdTATMMP was incubated with different doses of MMP-9 (0, 0.1, 1, and 10 ng/µl) at 37 °C for 2 h, previous to cell infection. $^*p < 0.05$, $^{**}p < 0.01$.

maximal MMP-9 concentration tested (Fig. 2A). Similar data was observed by incubation with MMP-2 protein, in agreement with the MMP linker being recognized by the two metalloproteases (data not shown). Moreover, no differences on AdTL transduction efficiency were observed by pre-treatment with MMP-9, highlighting the specificity of the effect to the TATMMP fiber modifications (Supplementary Fig. S2). Viral entrance of AdTATMMP was independent of CAR expression, in line with the fiber genetic modifications introduced, since AdTATMMP cell transduction was not inhibited by the presence of increasing concentrations of an inactivated virus with wild-type fiber, whereas competitive inhibition of transduction was observed with the inactivated wild type fiber virus and AdTL (Supplementary Fig. S3).

3.2. AdTATMMP transduction efficiency is superior to AdTL in neoplastic cells and cancer associated fibroblasts in vitro

We then sought to evaluate the transduction efficiency of AdTATMMP compared to the control AdYTRGE in a battery of cells expressing variable levels of MMP-2 and MMP-9 (Supplementary Fig. S4A). The control CAR-ablated virus AdYTRGE had a low capacity to infect cells. However, AdTATMMP fiber-modified virus rescued the infectivity in all the cell lines tested (Fig. 2B). Interestingly, AdTATMMP showed increased transduction compared to the wild-type fiber virus AdTL in cells that have limited infectivity with AdTL (PANC-1 and NIH-3T3). In contrast, in cells highly susceptible to wild-type fiber mediated-transduction, the transduction efficiency achieved by AdTATMMP was similar to AdTL (in HT1080) or lower (in RWP-1) (Fig. 2B and C). Expression of MMP-2 and MMP-9 in these two cell lines showed high levels of MMPs in HT1080, but moderate in RWP-1 (Supplementary Fig. S4A). Thus the efficiency of AdTATMMP transduction correlated with endogenous MMP content in the studied cell lines.

Pancreatic tumors are characterized by a highly abundant stroma composed of extracellular matrix and a heterogeneous population of CAFs, PSCs, endothelial and inflammatory cells [21]. CAFs and PSCs present tumor promoting capabilities and produce fibronectin and collagens, thus becoming important barriers to the distribution of oncolytic adenovirus. In fact, oncolytic adenoviruses active in CAFs have been shown to improve tumor virotherapy [22]. Based on these findings, we set out to investigate the susceptibility to adenoviral infection of CAFs and PSC cells from 3 different patients with pancreatic ductal adenocarcinoma (PDAC). Cell transduction was significantly increased by AdTATMMP compared to AdTL ranging from 35-fold to 108-fold (Fig. 3A and B). This result was in line with the abundant or moderate expression of MMP-2 and MMP-9 in CAFs and PSCs, respectively (Supplementary Fig. S4B).

3.3. AdTATMMP exhibits enhanced tumor transduction in mouse models of PDAC

To evaluate the tumor-targeting selectivity of AdTATMMP, wild type (wt) and transgenic Ela-myc mice, which develop tumors in the pancreas, were intravenously injected with a single dose of AdTL, AdYTRGE, or AdTATMMP at 5×10^{10} vp/mouse. At viral injection, transgenic mice displayed ductal and acinar tumors with abundant stroma [16] and a pancreatic volume that was 2-fold that of a normal pancreas (Supplementary Fig. S5). Four days later, bioluminescence was measured in the liver and the pancreas. As shown in Fig. 4A, a reduction in the liver transduction was observed with AdYTRGE and AdTATMMP viruses compared to AdTL, in agreement to their impairment to bind coagulation factor IX and CAR. Of notice, transduction of the pancreatic tissue in wt animals was highly impaired by both AdYTRGE and AdTATMMP compared to AdTL. However, pancreatic tumors from Ela-myc mice were efficiently transduced with AdTATMMP but not with AdYTRGE showing the in vivo tumor targeting capacity of AdTATMMP (Fig. 4B). Furthermore, tumor selectivity of AdTATMMP could also be observed upon intraductal delivery of AdTL and AdTATMMP in wt and Ela-myc mice. AdTL injection in wt mice resulted in a large number of pancreatic cells staining for GFP that was reduced in the tumorigenic pancreas of Ela-myc mice. On the contrary, a low number of GFP stained cells were detected in the pancreas of wt mice receiving AdTATMMP, whereas increased GFP staining was observed in Ela-myc tumors (Fig. 4C).

Since AdTATMMP efficiently transduced both neoplastic and CAF cells in vitro, we next assessed its in vivo tumor targeting activity in a mixed xenograft model generated from tumor cells (PANC-1) and human fibroblasts (CAF-25). Although human fibroblasts are unable to survive long-term, at least a 19-day long survival has been reported in some models [22] and this transient presence can promote tumor growth [23]. In this line, our mice model of PANC-1 + CAF-25 intrapancreatic tumors showed a 2.6-fold increased volume 30 days post-cell inoculation, when compared to PANC-1 tumors (Supplementary Fig. S6). Since the selectivity of AdTATMMP relies on the expression of tumor metalloproteases, we compared the expression of MMP-2 in PANC-1 + CAF-25 tumor areas versus tumor-free areas from the pancreas of the same animals. RT-qPCR analysis revealed a statistically significant increase of MMP-2 mRNA levels in tumor areas (Fig. 5A). To evaluate the selectivity of AdTATMMP in this mouse model, AdTL and AdTATMMP were intraductally injected in the pancreas of mice bearing PANC-1 + CAF-25 tumors and luciferase activity was measured four days later. Interestingly a 9-fold increase in AdTATMMP luciferase activity was measured in tumor areas compared to AdTL. Of note, the ratio of luciferase activity between tumor and tumor free areas was of 25-fold for AdTATMMP, whereas AdTL showed an inverse ratio, with a reduced luciferase activity in PANC-1 + CAF-25 tumors versus normal pancreas (Fig. 5B). These results highlight the tumor targeting capacity of AdTATMMP.

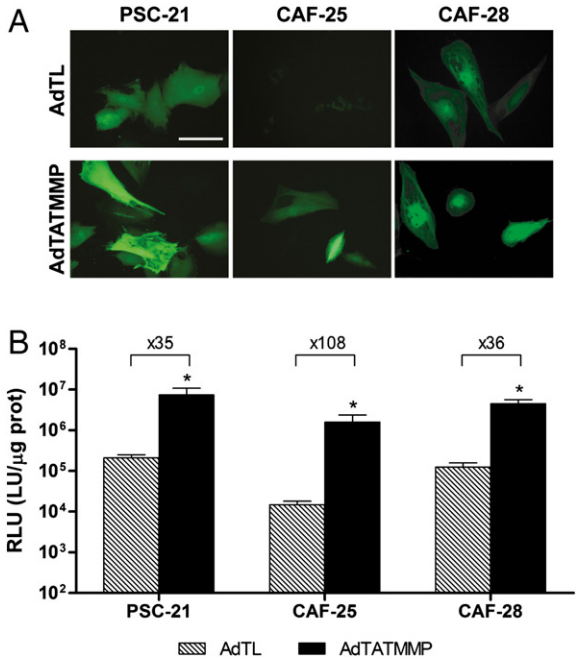

Fig. 3. AdTATMMP transduction efficiency in CAF and PSC cell lines. A total of 10^3 cells were seeded in triplicate and infected with either AdTL or AdTATMMP at 10^4 vp/cell. GFP and luciferase expression were measured three days later. A, representative GFP images. Scale bar = 200 μm. B, luciferase expression normalized to total protein levels. Results are represented as the mean ± SEM of at least 3 independent experiments. AdTATMMP fold change infection with respect of AdTL is shown above graph bars. *p < 0.05, **p < 0.01.

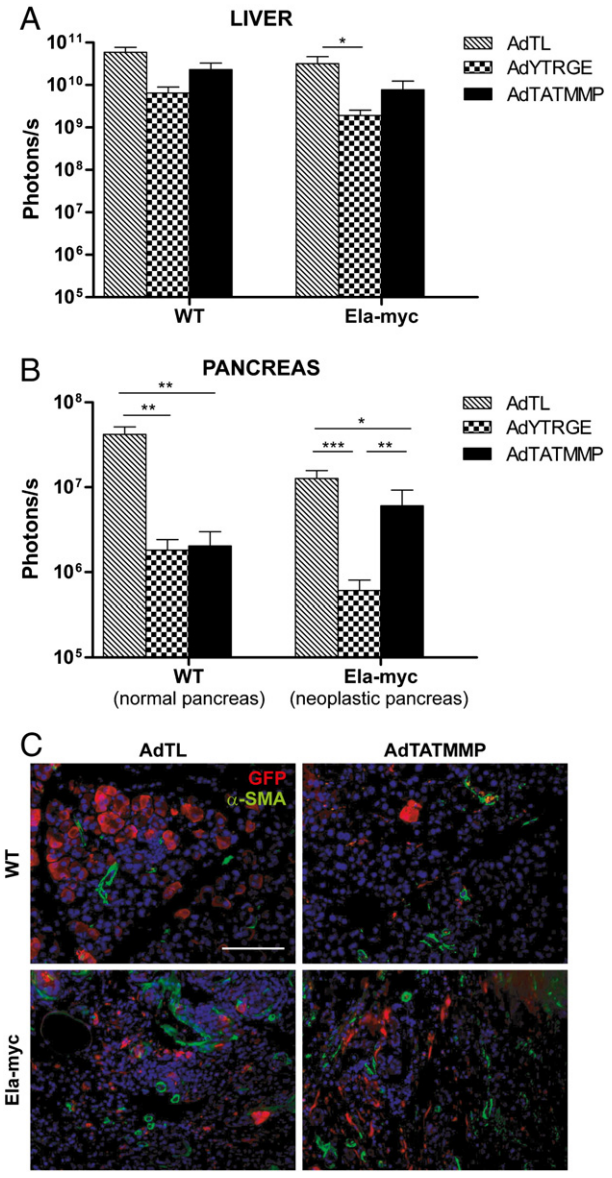

Fig. 4. AdTATMMP transduction efficiency in transgenic Ela-myc mice. A,B, 5×10^{10} vp of AdTL (n = 8), AdYTRGE (n = 6), or AdTATMMP (n = 8) were intravenously administered to Ela-myc and wt mice. Four days later, bioluminescence emission of liver and pancreas was measured. Luciferase expression was quantified from captured images and expressed as photons/s. C, representative images of GFP and α-SMA expression in pancreas of wt and Ela-myc mice intraductally injected with 5×10^{10} vp of AdTL or AdTATMMP. Scale bar = 40 μm. *p < 0.05, **p < 0.01, ***p < 0.001.

3.4. Ad^{RC}MMP shows significant oncolytic activity and triggers strong antitumoral response in orthotopic xenografts

To test whether the TATMMP fiber modification could provide oncolytic selectivity, we generated a replication competent adenovirus with the engineered fiber from AdTATMMP and designated this virus as Ad^{RC}MMP (Fig. 6A). Tumor cell cytotoxicity of Ad^{RC}MMP virus was first explored in vitro in HT1080, PANC-1, RWP-1, and in CAF-28 cells and compared to Adwt. The cytotoxic effect triggered by Ad^{RC}MMP virus was significantly higher than that of Adwt at all the viral doses tested in HT1080, PANC-1 and CAF-28, although less activity was detected in RWP-1 cells (Fig. 6B and C). Furthermore, the analysis of viral genomes in PANC-1 cells infected with Ad^{RC}MMP showed a significant increase of 10 and 33-fold at 3 and 5 days of viral production, respectively, when compared to Adwt. In contrast, reduced viral genomes were detected in RWP-1 cells infected with Ad^{RC}MMP (Fig. 6D). These results are in agreement with the transduction efficiency experiments shown in Fig. 2C, and with the CAR/MMP ratio. To evaluate the antitumor activity of Ad^{RC}MMP virus, PANC-1 cells engineered to express the luciferase gene (PANC-1Luc) were coinjected with CAF-28 fibroblasts in the pancreas of nude mice. Twenty-one days after cell inoculation animals received intraductally either saline, Adwt, or Ad^{RC}MMP at 5×10^{10} vp/mouse and tumor growth was monitored. Tumor progression was attenuated with both treatments for the 42 days period of the study, as shown by the tumor luciferase activity follow-up (Fig. 6E). Animals receiving Ad^{RC}MMP showed a statistically significant reduced luciferase activity when compared to Adwt treated mice.

At sacrifice, tumors were excised and primary tumors and metastatic foci were analyzed. Animals treated with Adwt or Ad^{RC}MMP showed statistically significant reduced tumor volumes compared to the saline group. In 2 out of 10 Adwt and in 3 out of 8 Ad^{RC}MMP treated mice, tumors completely regressed (Fig. 6F). Animals from all groups displayed metastatic foci in the liver and diaphragm, although in the Ad^{RC}MMP group these foci were detected only in 10% of mice. Interestingly, Ad^{RC}MMP treated mice did not show any metastasis in the lungs, whereas they were detected in 30% of Adwt-treated animals and in 90% of the saline group (Fig. 6G).

3.5. TATMMP-fiber modified adenovirus displays improved toxicological profile after intraductal delivery but not after systemic delivery

Since Ad^{RC}MMP intraductal delivery into mice bearing pancreatic tumors showed good antitumoral response, we sought to evaluate the toxicological profile of Ad^{RC}MMP in immunocompetent mice. As an indication of the pancreatic toxicity we analyzed the activity of the pancreatic enzymes amylase and lipase in serum of saline, Adwt, and Ad^{RC}MMP treated mice. A significant increase in both amylase and lipase

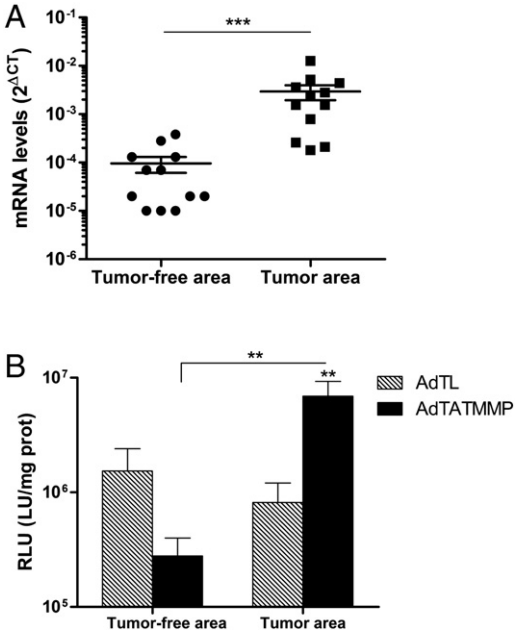

Fig. 5. AdTATMMP transduction efficiency in mixed orthotopic pancreatic tumors. A total of 3.5×10^5 PANC-1 plus CAF-25 cells (ratio 10:1) were orthotopically injected into the pancreas of nude mice. When tumors achieved a mean volume of 100 mm^3, animals were randomized and injected intraductally with 5×10^{10} vp of AdTL ($n = 6$) or AdTATMMP ($n = 6$). Animals were sacrificed four days later, the pancreas was obtained, and tumor areas were isolated from tumor-free areas. A, RT-qPCR expression of MMP-2 from tumor-free areas and PANC-1/CAF-25 tumors. B, luciferase activity in tissue extracts from tumor-free and tumor areas. *$p < 0.05$,** $p < 0.01$, ***$p < 0.001$.

was observed upon Adwt administration. In contrast, Ad^{RC}MMP showed pancreatic enzyme levels similar to saline treated mice (Fig. 7A). These data indicated that the intraductal delivery of Ad^{RC}MMP showed a good safety profile. These results are in line with the reduced transduction efficiency of non-tumoral pancreas by AdTATMMP after intraductal delivery (Fig. 4C and 5B).

To explore for the potential benefits of systemic delivery of TATMMP-fiber modified viruses and to discard their potential to accumulate in specific organs triggering undesired toxicities, we performed biodistribution studies with AdTL, AdYTRGE, and AdTATMMP reporter adenoviruses, and analyzed luciferase activity in a battery of organs. No preference for any organ by AdTATMMP was observed. In fact, AdYTRGE and AdTATMMP showed significantly decreased luciferase activity in liver and spleen, and a tendency to reduced levels in heart and lung. However similar transduction efficiency with all three viruses was observed in muscle

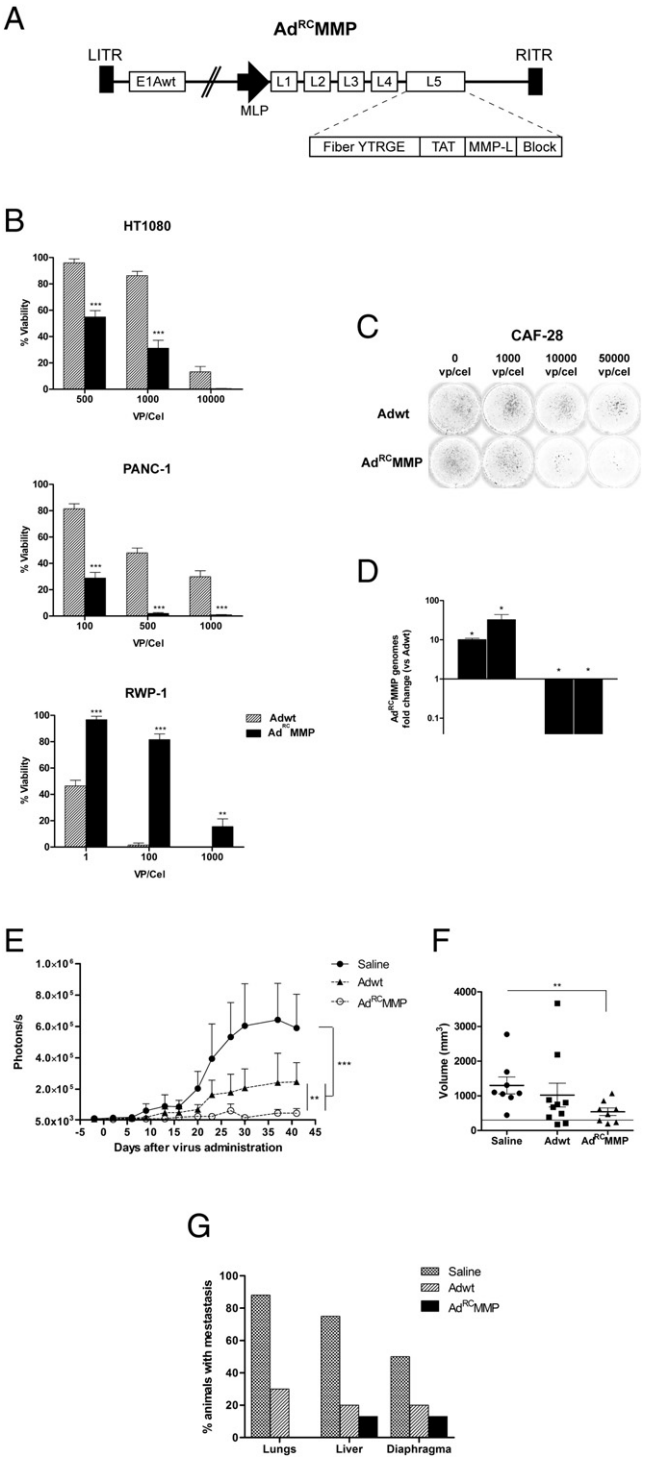

Fig. 6 Oncolytic effects of Ad^{RC}MMP in vitro and in PANC-1/CAF-28 tumor-bearing mice. A, schematic representation of Ad^{RC}MMP. B, a total of 3×10^3 cells were seeded in triplicate in a 96-well plate and infected with either Adwt or Ad^{RC}MMP at the indicated doses. Cell viability was measured 5 days later by an MTT assay. Results are represented as the mean ±SEM of at least 3 independent experiments. C, a total of 10^3 CAF-28 cells were seeded and infected as in A. Cell viability was measured 5 days later by methylene blue staining. D, PANC-1 and RWP-1 cells were seeded at 40,000 cells per well in duplicate in 24-well plates and infected with either Adwt or Ad^{RC}MMP at 50 vp/cell in the absence of FBS. Genomic DNA was extracted at 3 and 5 days post-infection and viral yield determined by qPCR. Results are represented as the mean ± SEM of at least 3 independent experiments. E–G, PANC-1-Luc/CAF-28 orthotopic tumors were generated in nude mice. Animals were randomized and intraductally injected with saline ($n = 8$), 5×10^{10} vp of Adwt($n = 10$) or Ad^{RC}MMP ($n = 8$), 21 days after cell injection. Tumor growth was monitored by in vivo luciferase emission. Animals were sacrificed 42 days after virus administration. E, luciferase quantification of bioluminescent emission images from saline, Adwt, and Ad^{RC}MMP treated mice. Results are expressed as photons/s. F, pancreatic volume at animal sacrifice, the line corresponds to the pancreatic volume of wt mice. G, presence of lung, liver, and diaphragm metastasis at sacrifice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

and kidney (Fig. 7B). Despite a reduction in liver uptake this was not sufficient to completely abrogate the liver damage triggered by viral-driven gene expression, in this case mainly due to the reporter luciferase, since serum ALT and AST were significantly increased in AdTL, AdYTRGE and AdTATMMP treated groups with respect to saline treated mice (Fig. 7C).

4. Discussion

PDAC remains a neoplasia highly refractory to conventional treatments [24]. Although some clinical improvement has been achieved with FOLFIRINOX [25] and more recently with the combination of nab-paclitaxel and gemcitabine [26], there is a need for more effective therapeutic approaches.

Oncolytic virotherapy is a promising anticancer therapy. Nevertheless, limited results from clinical trials indicate a need for improvement. One of the obstacles of adenovirus virotherapy with the most commonly used adenovirus type 5 is that the transduction of tumor cells is limited because CAR expression is often lost in neoplastic cells during tumorigenesis. Another hurdle of virotherapy is that CAFs, the major cell type in the tumor stromal compartment, are resistant to adenovirus infection. Adenoviruses modified with TAT peptides to broaden tropism have shown high transduction efficiency and enhanced therapeutic effects. However combination with other targeting strategies, such as the use of tumor-specific promoters driving viral genes, was needed to achieve tumor selectivity [27]. The insertion of peptide ligands into the HI loop or at the C-terminus of the Ad5 fiber has resulted in an enhanced infectivity, as in the case of the RGD peptide insertion [4]. However, structural constrains have often limited the success of some fiber modifications [28].

In the present study we have modified the tropism of replication competent adenovirus type 5 in a tumor-selective approach, by the modification of the adenovirus fiber. We engineered the C-terminal end of the adenoviral fiber with a TAT-like peptide sequence followed by a metalloprotease cleavable linker and a glutamic stretch. This was performed in a mutated adenoviral fiber background that was CAR and FIX binding ablated. We hypothesized that the TAT-like peptide will increase cell transduction, as previously reported [3,5] and the MMP-linker-blocking system will confer tumor selectivity. We speculated that the glutamic stretch would block the positively charged TAT-peptide, limiting its activity. Upon protease cleavage, the glutamic stretch would be released from the viral particles and the TAT-peptide would be exposed and fully active. Such a blocking strategy is novel, although polyanionic peptides inhibiting the entry of closely opposed cell penetrating peptides have been previously developed and they have shown that if the inhibitory moiety diffuses away, the activity is restored [29].

The success of our strategy requires the cleavage of the adenoviral fiber by MMP to expose the TAT-like peptide that facilitates viral entry. Our data shows that upon incubation of AdTATMMP with metalloproteases, viral transduction efficiency was increased in a dose-dependent manner, suggesting that MMP cleavage was successful exposing the TAT-like peptide and triggering viral entry. The concept of MMP-dependent virus activation has been successfully tested in enveloped viruses whose natural transduction process often requires cleavage of surface glycoproteins by intracellular proteases [12,30,31]. To our knowledge, the current work is the first report demonstrating that MMP-dependent virus transduction is compatible with genomic fiber modification, facilitating the development of entry-targeted adenoviruses.

AdTATMMP rescued the control CAR-ablated virus (AdYTRGE) infectivity in all the cancer cells tested, and significantly enhanced transduction of cell lines refractory to adenovirus infection (compared to the wild-type fiber AdTL). CAFs and PSCs from the tumor microenvironment showed higher transduction with AdTATMMP than with the CAR-binding AdTL virus. This was not surprising since in CAF cells MMP-2 and MMP-9 were highly expressed, in line with data reporting that

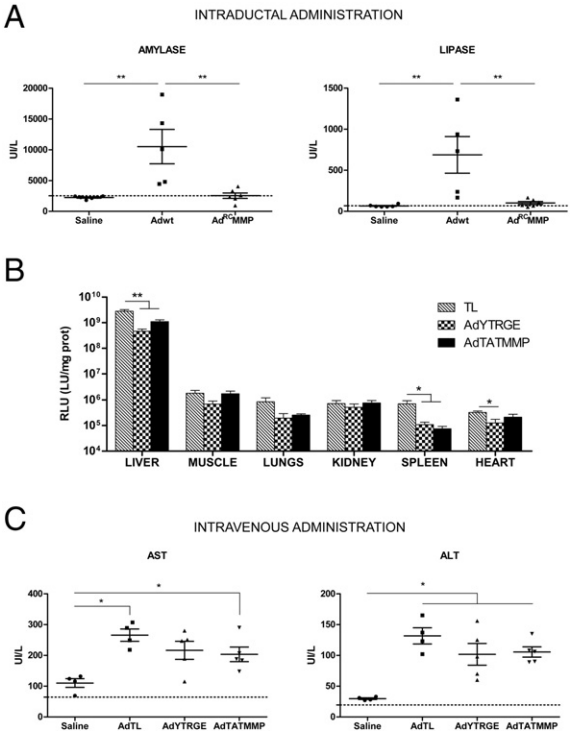

Fig. 7. Biodistribution and toxicity assays of TATMMP-fiber modified adenovirus. A. Saline (n = 6) or 5×10^{10} vp of Adwt (n = 5) or Ad^{RC}MMP (n = 6) were intraductally administered to wt C57BL/6 mice. Animals were sacrificed four days later and blood samples were obtained. Serum amylase and lipase were determined for pancreatic toxicity assessment. B–C. Saline (n = 4) or 5×10^{10}vp of AdTL (n = 4), AdYTRGE (n = 5) or AdTATMMP (n = 5) were intravenously administered to wt C57BL/6 mice. Animals were sacrificed four days later and blood and organ samples were obtained for posterior analysis. B, Luciferase activity in tissue extracts from isolated organs. C, serum AST and ALT levels. Dashed lines correspond to the reference values for C57BL/6 mice.*$p < 0.05$, **$p < 0.01$.

pancreatic tumors are highly enriched in MMPs, with MMP-2 and MMP-9 among the most abundant proteases in PDAC [32]. Moreover, the AKGLYK linker in AdTATMMP may also serve as a substrate for other proteases also contributing to the selective infectivity [33]. The possibility that adenoviruses could efficiently act on cells from the tumor microenvironment may be crucial to improve virotherapy-based treatments, and of special relevance in pancreatic cancer since these tumors present extensive desmoplasia with high content of CAFs and PSCs [34]. We show that AdTATMMP transduces tumors 9-fold more efficiently than AdTL in a mouse model generated by the co-inoculation of tumor cells and CAFs. This was a highly aggressive model where CAFs promote pancreatic tumor growth or tumor initiation, since PANC-1 + CAF tumors had larger volumes than PANC-1 tumors. In fact, there are compelling evidences that tumor microenvironment is an important regulator of tumor growth, tumor initiation, and metastasis [35]. Moreover, the contribution of CAFs to tumorigenesis has also been described for breast tumors [36]. In the PANC-1 + CAF-25 mixed model, MMP expression was high in the tumors what triggered efficient activation of AdTATMMP thus facilitating tumor transduction.

AdTATMMP showed strong tumor selectivity in vivo, since low transduction of the pancreas of wt animals was observed when it was delivered intravascularly or through a locoregional route. AdTATMMP pancreatic transduction was similar to AdYTRGE and 60 times less than that of AdTL in wt mice. Of note, in pancreatic tumors from transgenic Ela-myc mice, AdTATMMP showed increased transduction whereas AdYTRGE and AdTL virus displayed reduced activity than in wt animals. This AdTL and AdYTRGE in vivo reduced tumor transduction could be

the consequence of an impaired viral spreading in tumors. Although such impairment would affect all viruses, the tumor MMP activation of AdTATMMP might compensate for it and result in increased AdTATMMP tumor transduction. This tumor-transduction selectivity was also observed in the PANC-1 + CAF mixed model, in which AdTATMMP showed 25-fold enhanced transduction in tumor versus non-tumor areas of the pancreas, while AdTL showed a tendency to display less activity in tumors than in non-tumor areas.

Intravascular delivery showed some degree of liver detargeting, but this was not sufficient to ameliorate liver damage. Although AdTATMMP contains mutations that blocked FIX binding in the blood, this was insufficient, since major hepatic infection would be mediated by interactions between hexon viral protein with the blood coagulation factor X [37] and with the scavenger receptor in Kupffer cells [38]. Further improvement of AdTATMMP detargeting might be obtained by introducing genetic modifications to ablate FX binding in the capsid hexon protein [39] and by the strategies aimed at blocking scavenger receptor adenovirus interaction [40].

The fiber modifications in AdTATMMP, when introduced into a replication-competent virus, Ad^{RC}MMP, confer an increased lytic activity, superior to that of Adwt in cancer and CAF cells, in line with its enhanced transduction efficiency. Accordingly, a superior in vivo therapeutic activity was observed with Ad^{RC}MMP when compared to Adwt in orthotopic xenografts derived from the mixed PANC-1 + CAF model. Antitumor activity, as well as reduced number of metastatic foci, was observed in Ad^{RC}MMP animals showing the beneficial outcomes of the treatment. Interestingly, a good toxicological profile with Ad^{RC}MMP was obtained when administered intraductally.

In conclusion we present a novel adenoviral tumor targeting approach that improves oncolytic antitumor response in pancreatic cancer models that may be attractive for clinical adenoviral virotherapy against PDAC.

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