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uPAR-controlled oncolytic adenoviruses eliminate cancer stem cells in human pancreatic tumors



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Abstract Pancreatic tumors contain cancer stem cells highly resistant to chemotherapy. The identification of therapies that can eliminate this population of cells might provide with more effective treatments. In the current work we evaluated the potential of oncolytic adenoviruses to act against pancreatic cancer stem cells (PCSC). PCSC from two patient-derived xenograft models were isolated from orthotopic pancreatic tumors treated with saline, or with the chemotherapeutic agent gemcitabine. An enrichment in the number of PCSC expressing the cell surface marker CD133 and a marked enhancement on tumorsphere formation was observed in gemcitabine treated tumors. No significant increase in the CD44, CD24, and epithelial-specific antigen (ESA) positive cells was observed. Neoplastic sphere-forming cells were susceptible to adenoviral infection and exposure to oncolytic adenoviruses resulted in elevated cytotoxicity with both Adwt and the tumor specific AduPARE1A adenovirus. In vivo, intravenous administration of a single dose of AduPARE1A in human-derived pancreatic xenografts led to a remarkable anti-tumor effect. In contrast to gemcitabine AduPARE1A treatment did not result in PCSC enrichment. No enrichment on tumorspheres neither on the CD133⁺ population was detected. Therefore our data provide evidences of the relevance of uPAR-controlled oncolytic adenoviruses for the elimination of pancreatic cancer stem cells.

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Introduction

Pancreatic ductal adenocarcinoma remains one of the most aggressive and devastating tumor malignancies with an overall 5-year survival rate of less than 5% (Kern et al., 2011). The only potentially curative treatment is the complete surgical resection of the tumor. Nevertheless, even after the complete tumor resection disease progression is often described. Under such circumstances identification of therapies that can target both bulk cancer cells and tumor

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initiating cells is important in improving pancreatic cancer treatment.

Recent work in pancreatic cancer biology has identified a distinct subpopulation of cells termed cancer stem cell (CSC) that may represent an integral part of the development and perpetuation of the tumor. Cells bearing stem cell properties from human adenocarcinoma have been defined by the expression of specific cellular markers, their self-renewal capacity, differentiation ability and *in vivo* tumorigenicity. In pancreatic cancer the subpopulations of CD44⁺/CD24⁺/ESA⁺ (Bednar and Simeone, 2009; Li et al., 2007, 2009); CD133⁺ (Hermann et al., 2007; Lonardo et al., 2011) or ALDH1⁺ (Rasheed et al., 2010) have been identified to fulfill all these criteria. Emerging evidences indicate that pancreatic cancer stem cells are resistant to chemotherapy and radiation suggesting that such cells may be the source of the virtual relapse of pancreatic cancer (Balic et al., 2012). Therapeutic modalities leading to the elimination of CSC are an opportunity for the effective treatment of pancreatic cancer.

Oncolytic adenoviruses are very promising anticancer agents. They are engineered to specifically target, replicate in and destroy cancer cells (Aghi and Martuza, 2005; Fillat et al., 2010; Friedman et al., 2012; Short and Curiel, 2009). Oncolytic adenoviruses targeting the abnormal Rb/p16 pathway have been shown to be able to kill brain and breast cancer initiating cells (Eriksson et al., 2007; Jiang et al., 2008). Furthermore, oncolytic adenoviruses driven by the tissue-specific promoters multidrug resistance (*mdr*) and cyclo-oxygenase 2 (*Cox-2*) have been shown to be active in breast cancer initiating cells (Bauerschmitz et al., 2008).

Recent studies in small cell lung cancer identified cells with stem-like properties being positive for uPAR gene expression (Gutova et al., 2007; Qiu et al., 2012). As we have previously shown uPAR-controlled adenoviruses are highly active in pancreatic cancer cell lines and exert strong antitumor and anti-metastatic effects in xenografts (Huch et al., 2009). In the current study we sought to explore the ability of uPAR controlled oncolytic adenoviruses to act against pancreatic CSC (PCSC). We used two primary patient xenograft models to characterize pancreatic cancer stem cells and determined the effects of AduPARE1A on PCSC cells and compared to those of gemcitabine, the most common chemotherapeutic agent used in pancreatic cancer treatment. We found that PCSC are susceptible to adenoviral transduction and are sensitive to adenovirus mediated cell death by AduPARE1A oncolytic adenovirus. *In vivo* treatment with a single AduPARE1A viral administration triggered significant reduction in tumor progression. In contrast to gemcitabine treatment, AduPARE1A did not increase the PCSC population. Thus, our results show that AduPARE1A shows efficacy in suppressing pancreatic cancer stem cells and could be a beneficial therapy to treat pancreatic cancer patients.

Materials and methods

Preparation of single-cell suspensions from human PDAC tumors

Human CP15 and CP13 adenocarcinoma surgical samples were obtained and implanted into the pancreas of immunodeficient mice as previously described (Perez-Torras et al., 2011). CP15

and CP13 were selected from a tumor platform of 11 PDAC specimens. Tumorgrafts retained original morphology of the human primary tumor at the generations used in the current study. To obtain single-cell suspensions, CP15 and CP13 tumor fragments, from fresh or frozen tissue, were cut into small pieces and minced completely and then an enzymatic dissociation was applied using 200 U/ml ultrapure collagenase IV (Sigma, Freehold, NJ) in DMEM–F12 medium and incubated at 37 °C for 2 to 3 h. The specimens were further mechanically dissociated every 15 to 20 min by pipetting. At the end of the incubation, cell suspension was washed twice with DMEM–F12 supplemented with 10% of heat inactivated fetal bovine serum (FBS). Then, cells were filtered through a 70- μ m filter, collected in a fresh 50-ml conical tube and adjusted to a final volume of 15 ml per tube. In order to deplete death cells or tissue debris, tumor cell suspension was separated by density centrifugation using Ficoll-Paque Plus. Cell suspension and Ficoll layers were centrifuged at 500 \times g for 30 min with the brakes turned off. Dissociated cells were transferred to a fresh 50-ml conical tube. Fresh DMEM–F12 supplemented with 10% FBS was added to the cell suspension to dilute it 1:3. Cells were filtered through a 40- μ m filter and collected by centrifugation at 450 \times g for 10 min. The cell pellet was resuspended in 1 ml of DMEM–F12 supplemented with 0.4% FBS or in PBS/2% BSA according to the procedure.

Human pancreatic tumorsphere formation and culture conditions

To generate tumorspheres, single-cell suspensions from CP15 and CP13 tumors were cultured in DMEM–F12 supplemented with 0.4% FBS, Gibco, Life Technologies), 2 mM L-glutamine (Gibco, Life Technologies), 10 U/ml penicillin and 10 μ g/ml streptomycin (Gibco, Life technologies), 20 μ g/ml gentamicin (Gibco, Life Technologies), B27 1 \times (Gibco, Life Technologies), 5 μ g/ml insulin (insulin solution from bovine pancreas, I0516 Sigma-Aldrich), 20 ng/ml of recombinant human epidermal growth factor (HuEGF, Invitrogene, Life Technologies), and 20 ng/ml basic fibroblast growth factors (bFGF, BD Bioscience) at 37 °C and plated onto 24 multiwell plates (Lab-Tek, Nunc) at a density of 5.000–10.000 cells/well. These plates were previously coated with 10 mg/ml Poly-HEMA (Sigma-Aldrich). The medium was designated as tumor-initiating cell medium or TIC medium. Cells were cultured in TIC medium for at least 1–2 weeks or until the appearance of non-adherent spherical clusters. Spheres were enzymatically dissociated (Trypsin-EDTA), and sub-cultured in TIC medium for several passages before initiating the experimental assays. To allow differentiation, dissociated sphere cells were plated under standard medium supplemented with 10% FBS.

Flow cytometry analysis

Cells were resuspended in PBS 2% BSA and incubated for 30–45 min at room temperature with the corresponding antibodies. Then samples were washed twice with PBS 2% BSA, resuspended in PBS 2% BSA containing DAPI (4',6-diamidino-2-phenylindole, Sigma) at 1 μ g/ml and incubated for 15 min on ice. The antibodies used were: anti-CD133-APC (clone CD133/1 (AC133), Miltenyi Biotec), APC-H7 mouse anti-human CD44 (clone G44-26 (also known as C26), BD

Pharmingen™), PE mouse anti-human CD24 (clone ML5, BD Pharmingen™), PerCP-Cy5.5 mouse anti-human CD326 (EpCAM) (clone EBA-1, BD Pharmingen™), anti-H2K-FITC (H100-27.R55, Miltenyi Biotec) and appropriate isotype-matched control antibodies. In all the experiments using human xenograft tissue, infiltrating mouse cells were eliminated by discarding H2K (mouse histocompatibility class I) cells during flow cytometry, as previously described (Li et al., 2007). Dead cells were eliminated by using the viability dye DAPI. Flow cytometry was done using a BD™ LSR II flow cytometer or a BD FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed with CELLQuest Pro or FACSDiva software (Becton Dickinson). GFP-positive cells were quantified by flow cytometry (BD™ LRS II).

Adenoviruses

AduPARE1A and AdCMVGFPLuc have been previously described (Alemany and Curiel, 2001; Huch et al., 2009). Adwt, the wild-type adenovirus, was obtained from the ATCC (Manassas, VA). Replication-defective viruses and Adwt were propagated in HEK293 cells, and the oncolytic AduPARE1A virus was amplified in RWP1 cells. All viruses were purified by standard cesium chloride banding. The physical particle concentration (vp/ml) was determined by optical density reading (OD_{260}) and the plaque forming units (pfu/ml) were determined on HEK293 cells by the anti-hexon staining-based method (Cascante et al., 2007). AduPARE1A and Adwt presented an equal ratio of vp and pfu.

In vitro cytotoxicity assays

Cytotoxicity assays were performed by seeding 3,000 tumor initiating suspension cells per well in 48-well poly-HEMA coated plates and cultured in TICM. Cells were infected with serial dilutions of AduPARE1A and Ad5wt starting from 1×10^5 vp/cell to 0.001 vp/cell. Seven days later, plates were centrifuged and washed with PBS. Cell viability was measured using the MTT colorimetric assay. ID_{50} values were estimated from dose–response curves by standard nonlinear regression (GraFit; Erithacus Software, Horley, UK), using an adapted Hill equation.

RT-PCR

Total RNA from tumors and spheres was isolated using RNeasy (Qiagen, Hilden, Germany) following the manufacturer's instructions. Samples were treated with DNase (DNasefree, Ambion). Reverse transcription reaction was performed to generate cDNA using a Retroscript RT kit (Ambion). The obtained cDNA was PCR amplified with uPAR gene specific primers (Fwd: 5'-GCCTTACCGAGTTGTGTGT-3'; Rv: 5'-CATCCAGGCACTGTTCTTCA-3'). RT-PCR results were confirmed by two independent RT-PCR amplifications. E1A expression and uPAR expression were determined by quantitative PCR (Via™ 7, Applied Biosystem) using SYBR Green reagent and specific primers (E1A: Fwd:5'-ATCGAAGAGGTACTGGCTGA-3'; Rv:5'-CCTCCGGTGATAATGACAAG-3'; uPAR: Fwd: 5'-GCCTTACCGA GGTGTGTGT-3'; Rv:5'-CATCCAGGCACTGTTCTTCA-3'). β -Actin gene was used to normalize expression. β -Actin specific primers: (Fwd: 5'-CTGGAACGGTGAAGGTGACA-3'; Rv: 5'-GGGAGAGGAC TGGGCCATT-3').

Orthotopic human pancreatic cancer xenografts

Orthotopic human pancreatic cancer xenografts were generated as previously described (Jose et al., 2012; Kim et al., 2009). Briefly, a laparotomy incision in the left dorsal side of the mouse was performed to expose the spleen and the pancreas. Then, 3×10^5 CP15 or 4×10^5 CP13 tumor cells were injected into the pancreas of 8-week-old male athymic nude mice (Harlan Interfauna Iberica), in a final volume of 50 μ l. Abdominal muscle layer was closed with interrupted suture and the overlying skin was closed using Autoclips® (Stoelting Europe). Animals were randomly divided in two groups: saline, and AduPARE1A. Treatments were initiated when tumors reached a tumor volume ranging from 60 to 100 mm³, measured by dorso lateral palpation. A single dose of AduPARE1A (5×10^{10} vp/mouse) was intravenously injected. To assess the effect of the treatments, animals were euthanized 30 days after the initiation of the treatments. Pancreatic tumors were fractioned and frozen in OCT for histological analysis (Akura Finetek, Zoeterwoude) or in liquid nitrogen for molecular studies.

Orthotopic xenografts from pancreatic tumorspheres were generated as previously described (Jose et al., 2012; Kim et al., 2009) by the inoculation in the pancreas of single cell suspension after tumorsphere trypsin digestion.

Tumor growth studies

Pancreatic tumors were measured at the end of the experiments, and volumes were calculated according to the formula V (mm³) = $0.4 \cdot (\text{larger diameter} \cdot \text{smaller diameter}^2)$. Animal procedures met the guidelines of European Community Directive 86/609/EEC and were approved by the Local Ethical Committee.

Immunohistochemistry

Paraformaldehyde-fixed paraffin embedded tumors were obtained and 3 μ m paraffin embedded sections were stained with haematoxylin–eosin for morphological analysis. Immunofluorescence was performed in frozen tissue sections embedded in OCT by incubating with the anti-adenovirus 2/5 E1A antibody (sc-430, Santa Cruz Biotechnology Inc.). Alexa Fluor 633-labeled goat anti-rabbit IgG antibody (Molecular Probes, Life Technologies) was used as a secondary antibody. The nuclei were counterstained with 5 μ g/ml bis-benzimide (Hoechst 33342; Sigma) and visualized under a fluorescent microscope (Nikon Eclipse 50i, Spain). The fluorescent images were captured using a digital camera (CoolCube1, MetaSystems).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical significance was estimated with nonparametric U-Mann Whitney test or the parametrical *t*-test. A *P*-value of <0.05 was considered significant (*). Statistical analyses were carried out with Prism (version 5; GraphPad software).

Results

Characterization of cancer stem cells from human pancreatic tumors

As a first step towards the evaluation of the efficacy of oncolytic adenoviruses to eliminate cancer initiating cells in pancreatic tumors we characterized pancreatic cancer stem cells (PCSC) in patient samples derived from early passage human xenograft adenocarcinomas perpetuated in the pancreas and never expanded in vitro and designated as CP15 and CP13, (Table 1) (Perez-Torras et al., 2011). The expression of the cell surface markers CD133, and the combination of CD24, CD44 and ESA previously identified as characteristic of PCSC were analyzed by flow cytometry (Hermann et al., 2007; Li et al., 2007). Because an enrichment of CSC has been reported to occur upon chemotherapeutic treatments (Mueller et al., 2009; Hindriksen and Bijlsma, 2012), the study was performed in untreated xenografts and in xenografts from mice that received a weekly dose of gemcitabine (160 mg/kg) for five weeks (Fig. 1A). Gemcitabine treatment slowed tumor growth progression in both CP15 and CP13 models although statistically significant effects were only observed in CP15 tumors (Fig. 1B). Two weeks after the last gemcitabine dose stem cell markers were analyzed. Flow cytometric quantification of CD133 in dissociated tumor xenografts revealed noticeable differences in the two tumors, CP15 presented 37% of cells positive for the CD133 marker whereas 61% of CD133⁺ cells were detected in CP13. A significant increase in the number of CD133⁺ cells was observed in both CP15 and CP13 tumors upon treatment with gemcitabine (Fig. 1C). When examining the expression of CD24 CD44 and ESA cell surface marker combination, 0.6% and 1.1% of cells expressed the CD24⁺CD44⁺ESA⁺ combination in CP15 and CP13 tumors respectively. Gemcitabine treatment showed a tendency to increase the frequency of the CD24⁺CD44⁺ESA⁺ population in both CP15 and CP13 tumors (Fig. 1C). These data show that xenografts from CP15 and CP13 patient samples express cell surface markers of PCSC that are enriched upon a gemcitabine treatment.

Another well defining trait of cancer stem cells is their ability to grow in vitro as anchorage-independent colonies termed tumorspheres. In the current work we evaluated the capacity of isolated cells from CP15 and CP13 xenografts to grow as spheres and we measured the number and size of the spheres after the first and the fifth passage. Both tumors were successfully expanded to form tumorspheres and stably propagated for at least 20 passages. Comparing features in the tumorspheres of the two tumors, no differences were observed in the size of tumorspheres although a higher

number of spheres were observed in CP13 both in the first and the fifth passage. Consistent with an enrichment of PCSC upon gemcitabine treatment, the number and size of spheres were larger in tumorspheres expanded from gemcitabine treated xenografts. Differences were more remarkable in CP-15 tumors (3.8-fold increase in sphere number, 6.3-fold increase in the sphere size) (Fig. 2A). To further analyze if the tumorspheres exhibited the properties of stem cells we tested for their capacity to differentiate and to recapitulate the phenotype of the original tumor. Tumorspheres plated in standard medium displayed an epithelial phenotype typical of pancreatic cancer cell lines (Fig. 2B). Of notice the injection of single cell suspensions of tumorspheres into the pancreas of immunodeficient mice generated tumors that histologically resemble CP15 and CP13 patients' primary tumors with an early passage in nude mice (Fig. 2C).

Targeting cancer initiating cells with oncolytic adenoviruses

We tested the capacity of adenoviruses to transduce pancreatic cancer stem cells. Tumorspheres from CP15 and CP13 xenografts were infected with AdCMVGFPLuc reporter adenovirus and GFP expression was analyzed at different time-points. GFP expression was already evident at 24 h post-transduction and three days later more than 80% of the cells were positive for GFP (Supplementary Fig. 1). To evaluate the sensitivity of PCSC to adenoviral mediated lytic cell death, tumorspheres were incubated in the presence of different viral doses of wild type adenovirus (Adwt) and the AduPARE1A oncolytic adenovirus. A substantial cell killing effect was observed in tumorspheres after Adwt and AduPARE1A infection showing similar cytotoxicity in CP15 (ID₅₀: 104 ± 15.42 Adwt, 108 ± 15.01 AduPARE1A). CP13 tumorspheres displayed higher sensitivity to adenoviral cytolysis (ID₅₀: 71.72 ± 14.7 Adwt, 55.19 ± 7.74 AduPARE1A) (Fig. 3A). The increased sensitivity in CP13 vs CP15 was of 2.0-fold in AduPARE1A infected cells. This was in line with an increased expression of the uPAR gene in CP13 tumorspheres (Fig. 3B).

AduPARE1A oncolytic adenovirus treatment does not spare pancreatic cancer stem cells

To test the effect of AduPARE1A therapy in a clinically relevant setting, a single dose of AduPARE1A 5 × 10¹⁰ vp/mice was i.v. injected into mice bearing CP15 or CP13 tumors in the pancreas (Fig. 4A). Tumor volume was measured at the end of the experiment on day 30 after treatment was initiated. AduPARE1A treatment significantly

Table 1 Patient tumor data.

Model	Tumor staging	Nodal invasion	Perineural invasion	Vascular invasion	Metastatic recurrence	Histological features	k-Ras codon 12	p16 loss	p53 exons 4 to 9
CP13	T2N1	2/21	1	0	LFU	Well-defined glands	Yes	Yes	Exon 5
CP15	T3N1	15/28	1	1	Lymph nodes, liver, suprarenal	Irregular glandular pattern	Yes	Yes	wt

LFU: lost follow-up.

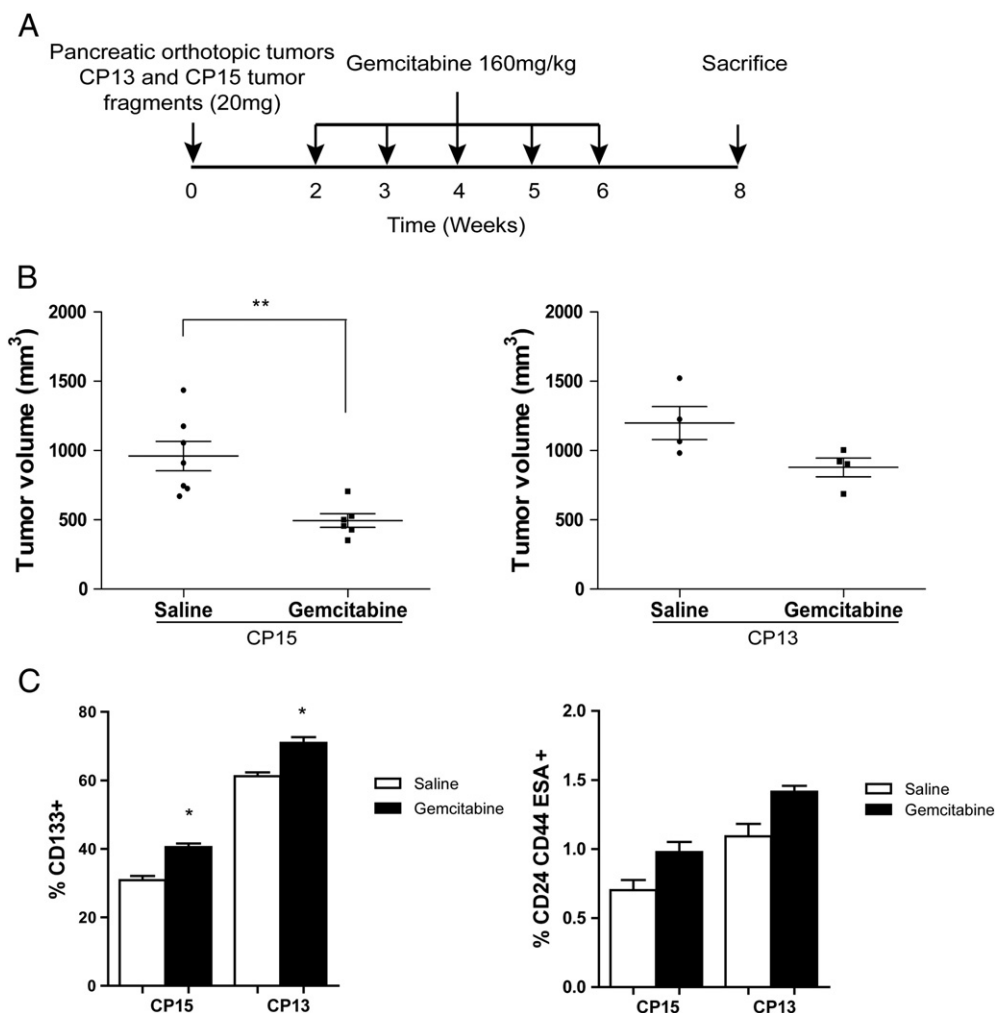


Figure 1 Analysis of pancreatic cancer stem cell markers in CP15 and CP13 patients derived xenografts treated with gemcitabine. A) Schematic representation of the treatment protocol. B) Analysis of the pancreatic tumor volumes (mm³) 6 weeks after treatment was initiated (n = 7, CP15; n = 4, CP13). C) Flow cytometry quantification of CD133 cancer stem cell marker (left) and the percentage of CD24 CD44 ESA positive cells (right) in dissociated treated tumor xenografts (n = 4). *p < 0.05, **p < 0.01.

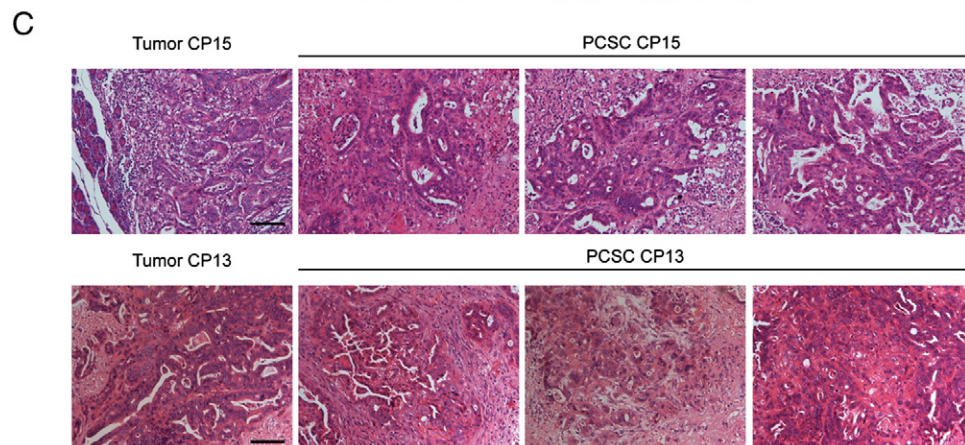
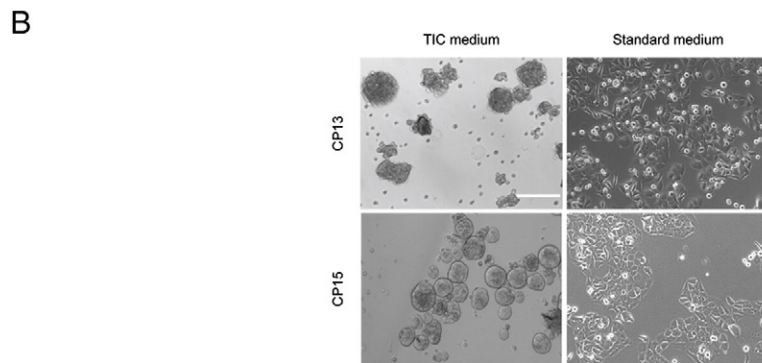
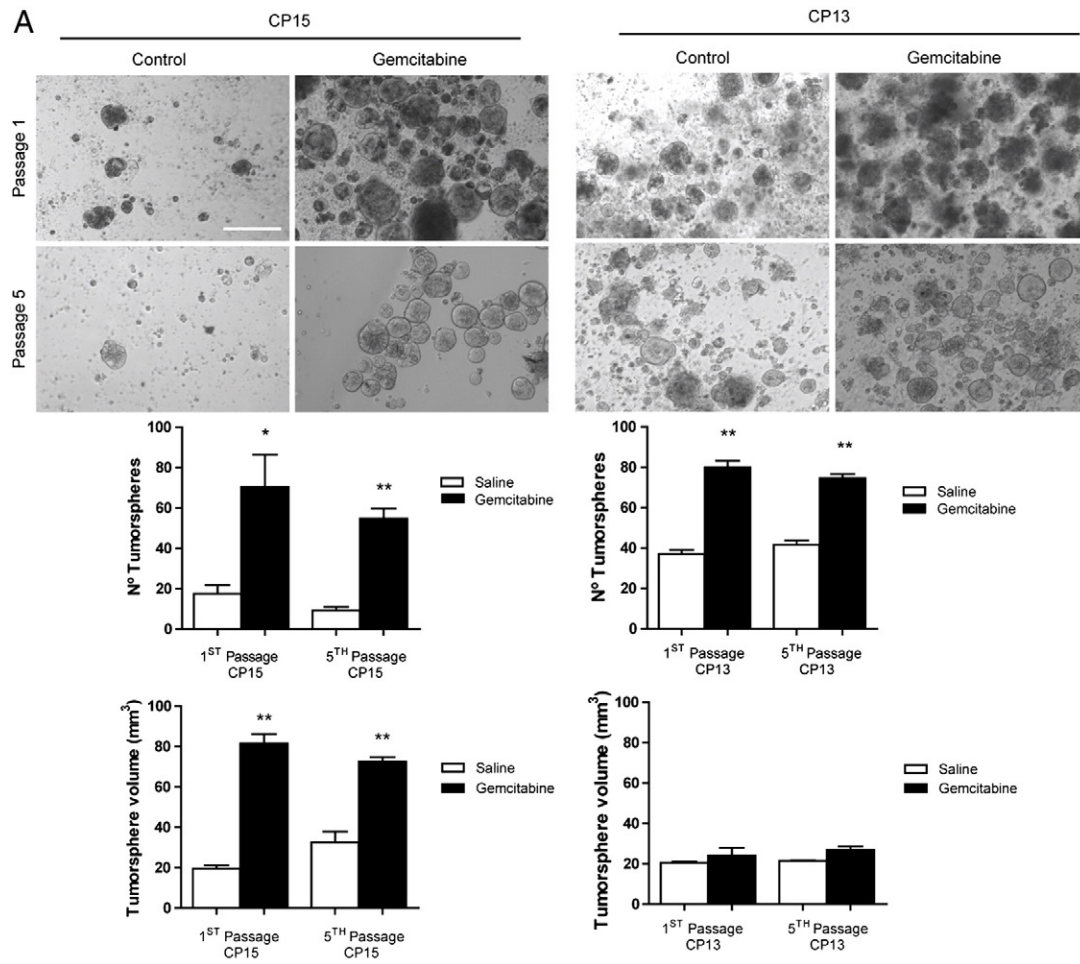
inhibited both CP15 and CP13 tumor growths (Fig. 4B). Evidence of viral replication was observed in treated tumors as shown by the expression of E1A (Figs. 5C, D). Current observations were limited to 30-days post-treatment due to the presence of life-threatening tumors in the CP15 control group. However, the detection of E1A gene expression in treated tumors suggests that AduPARE1A antitumoral effects could be more extensive if followed long term.

Having shown that gemcitabine treatment significantly increased the percentage of CD133⁺ cells and the number and size of tumorspheres from CP15 and CP13 treated tumors (Figs. 1C, 2A), we determined the effects of AduPARE1A treatment on the content of CD133⁺ cells and in tumorsphere formation capacity. Interestingly, in contrast to gemcitabine we did not identify any enrichment in the CD133⁺ cell population neither in CP15 nor in CP13 (Fig. 5A). Moreover AduPARE1A treated tumors showed a tendency to form less tumorspheres than saline injected tumors in CP15 and a significant smaller proportion of tumorspheres were formed in CP13 treated tumors, at the passage analyzed (Fig. 5B).

These data indicate that AduPARE1A triggers a strong antitumoral response in pancreatic tumors and does not enrich tumors on cancer stem cells.

Discussion

Recent studies in cancer therapy favor the idea that the disconnection between response rates and overall survival may rely in the failure to effectively targeting CSC populations. Furthermore survival of these long-lived cells in the presence of toxic compounds might facilitate for additional mutations and increased resistance. In the present study we have characterized PCSC from two patient-derived tumors and shown that, although gemcitabine treatment induced partial antitumor response, it leads to an increase in cells showing CSC-like properties. In contrast, a therapeutic approach based on the single intravenous administration of AduPARE1A oncolytic adenovirus triggered remarkable anticancer effects with no increase in the PCSC pool.



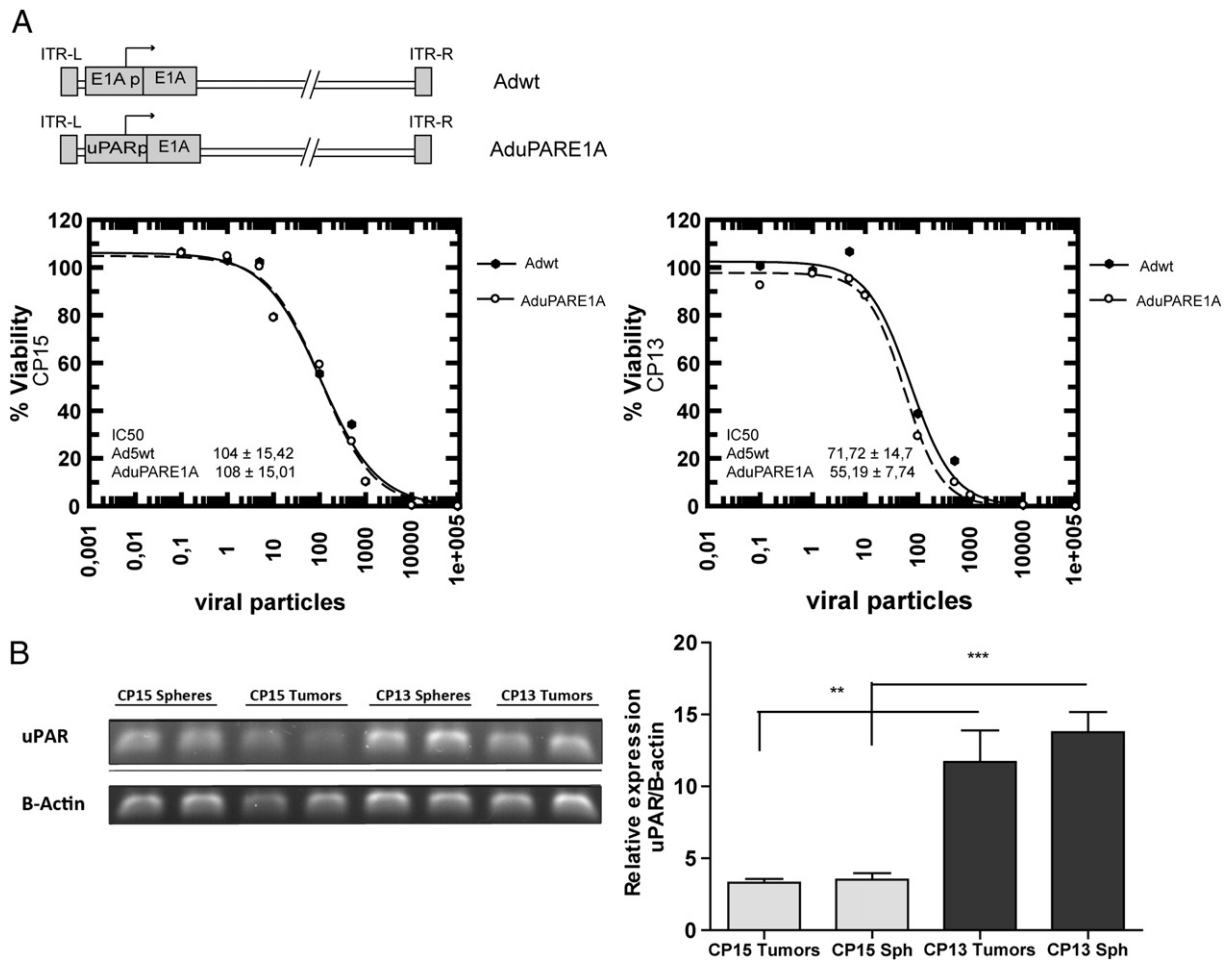


Figure 3 In vitro cytotoxic effects of AduPARE1A oncolytic adenovirus in CP15 and CP13 tumorspheres. A) Schematic representation of AduPARE1A and Adwt adenoviruses. MTT viability assay of CP15 (left) and CP13 (right) tumorspheres. A total of 3×10^3 cells/well were plated in triplicate and infected with a dose range of 10^{-3} to 10^5 MOIs of Ad5wt or AduPARE1A. Cell viability was measured 7 days later and is expressed as the percentage of absorbance of treated wells compared with that of mock-infected cultures. Dose-response curves and ID50 values were obtained by a standard non linear model based on the Hill equation. Data show a representative experiment ($n = 3$). B) RT-PCR analysis of uPAR gene expression in CP15 and CP13 tumors and tumorspheres. β -Actin was used as control for gene expression. Representative images and qRT-PCR.

In agreement with previous observations we demonstrate an enrichment in the CD133 population by gemcitabine treatment, but not in the CD24, CD44, and ESA positive-cells, suggesting that cancer stem cell chemoresistance, at least with gemcitabine, might differentially affect the PCSC repertoire (Mueller et al., 2009; Venkatesha et al., 2012). Gemcitabine treatment also increased the number of neoplastic sphere-forming cells and the size of the tumorspheres. To validate the tumorigenic potential of tumorspheres we demonstrate that the tumorspheres injected directly into the mouse pancreatic tail result in tumor formation that

histologically resemble the morphology of an early passage patient derived xenograft. These results support that tumorsphere derived from CP15 and CP13 tumors retain the features of tumor initiating cells in the pancreatic niche. All together, these suggest that gemcitabine treatment enriches in neoplastic cells with features of PCSC, in both CP15 and CP13 tumors.

Oncolytic adenoviruses seem like ideal candidates to target cancer initiating cells because they are cytotoxic and are not subject to the typical mechanisms of drug resistance, such as drug efflux pumps and defective apoptotic signaling. Indeed,

Figure 2 Analysis of tumorspheres from CP15 and CP13 patient derived xenografts treated with gemcitabine. A) Images of tumorspheres from CP15 and CP13 treated tumors formed from isolated cells that grew as anchorage-independent colonies. Quantification (number) and volume (mm^3) of tumorspheres formed from the CP13 and CP15 treated tumors at passages 1 and 5 (bar graphics) ($n = 6$). B) Images of CP15 and CP13 tumorspheres plated in TIC medium or standard medium. C) Hematoxylin and eosin staining of pancreatic tissue sections from CP15 and CP13 xenografts (left) or from CP15 ($n = 3$) and CP13 ($n = 3$) isolated tumorspheres injected into the pancreas of immunodeficient mice (right). Scale bars 100 μm . * $p < 0.05$, ** $p < 0.01$.

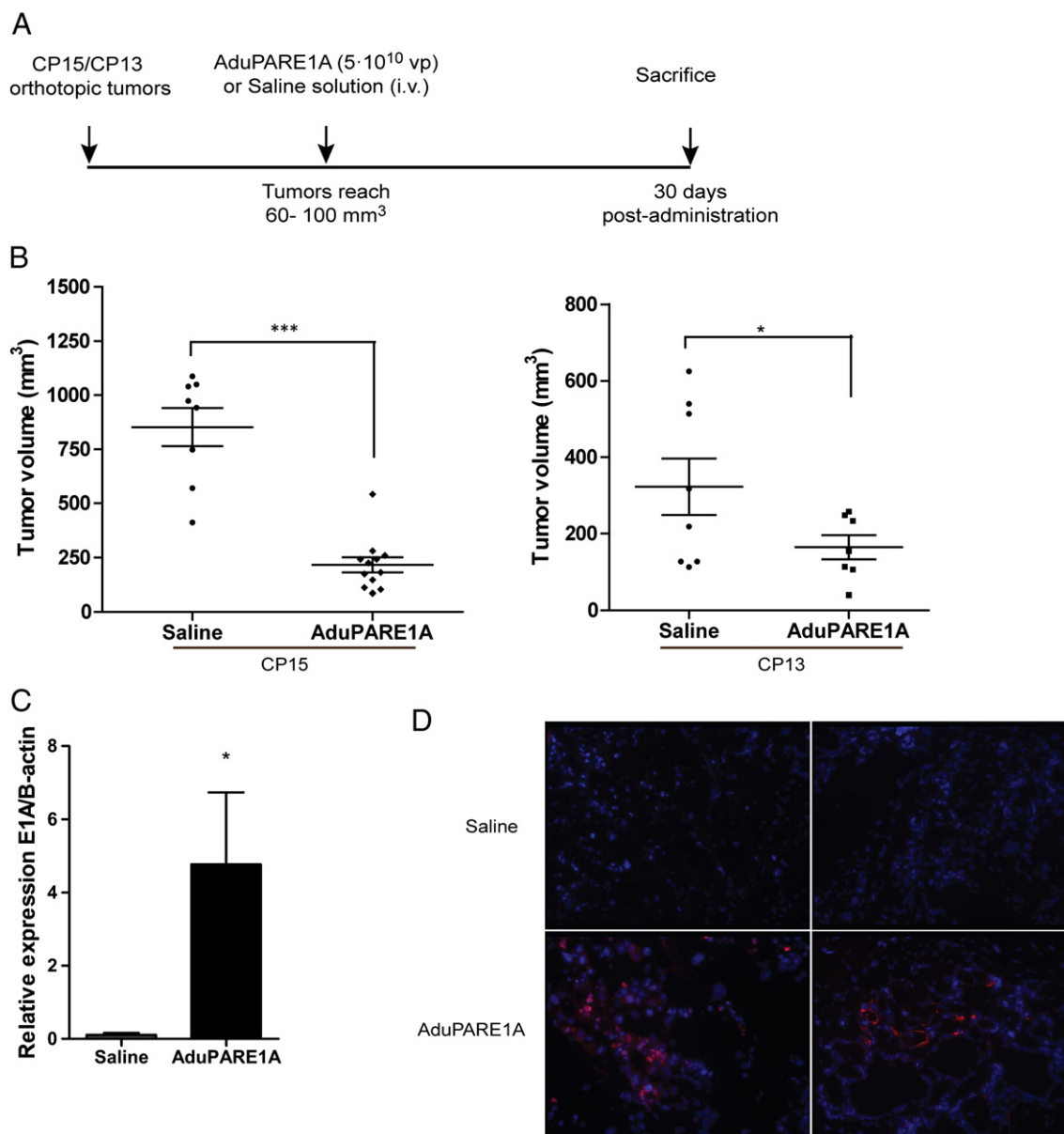


Figure 4 In vivo antitumor effects of AduPARE1A oncolytic adenovirus in CP15 and CP13 orthotopic tumors. A) Schematic representation of the treatment protocol applied in orthotopic tumors derived from CP15 or CP13 single cell suspension of pancreatic xenograft. AduPARE1A (5×10^{10} vp) or saline solution was intravenously administered to mice bearing CP15 or CP13 tumors in the pancreas ($n = 12$, $n = 7$) or ($n = 8$, $n = 8$) respectively. B) Pancreatic tumor volumes (mm³) in saline and treated tumors 30 days after virus injection are plotted as indicators of antitumor effect. C) qPCR analysis of adenoviral E1A gene expression in RT-PCR samples from CP15 treated tumors. β -Actin was used as reference gene for normalization ($n = 4$). D) Anti-E1A immunofluorescence of CP15 tumors 30 days after i.v. administration of AduPARE1A (5×10^{10} vp) or saline solution. Two tumors of each group are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

studies with oncolytic adenoviruses in breast and brain tumors suggest that they may be effective against CSC (Alonso et al., 2012; Bauerschmitz et al., 2008; Jiang et al., 2007). These viruses are often generated on the basis of its tumor selectivity to improve control of viral replication, resulting in diminished toxicity. The use of tissue specific promoters preventing the expression of E1A in non-target tissues is a useful strategy.

Here we show that uPAR promoter controlled adenoviruses are able to kill neoplastic-sphere forming cells in vitro with a similar or even with higher efficacy than wild type

adenoviruses. This is consistent with the observation that these cells endogenously express the uPAR gene, suggesting that the transcription factors acting on the regulatory sequences of the uPAR gene are present in the target cells and can activate the uPAR promoter. Importantly in vivo, AduPARE1A treated tumors were much smaller than mock-treated tumors, and had a similar proportion of CD133⁺ cells, and a smaller capacity to generate tumorspheres. This is in contrast to what was observed in gemcitabine treated tumors, where we observed an enrichment in the CD133

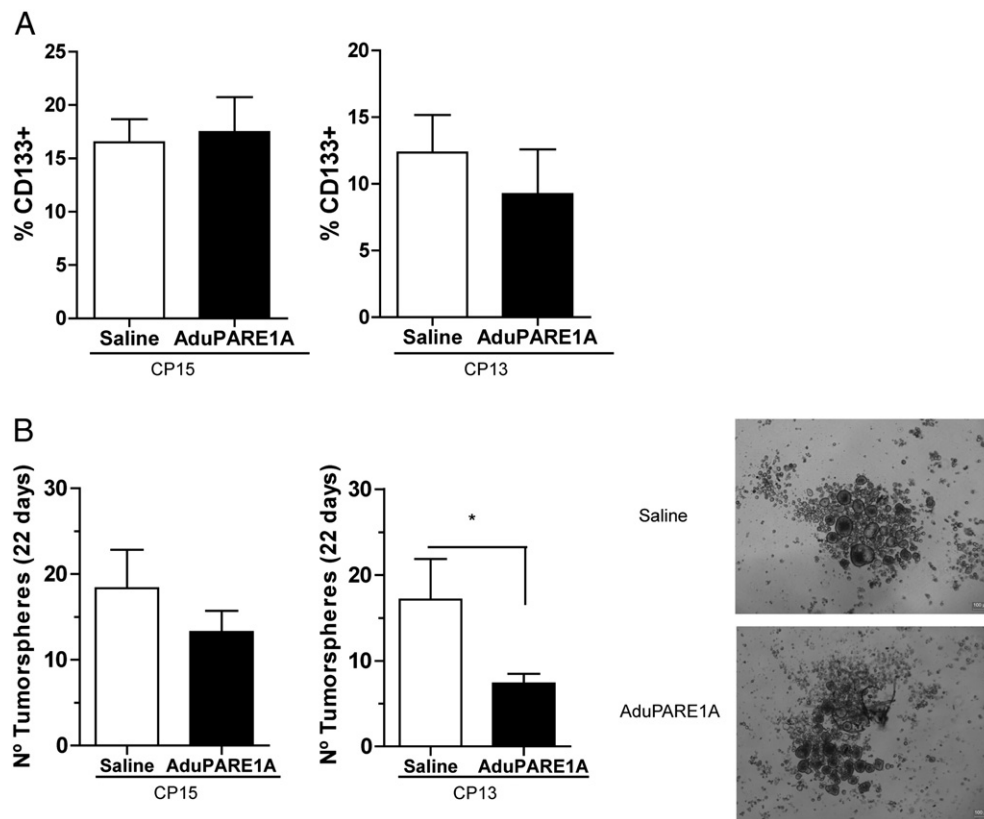


Figure 5 Analysis of pancreatic cancer stem cell features in CP15 and CP13 patient derived xenografts treated with AduPARE1A. A) Flow cytometry quantification of CD133 positive cells in CP15 and CP13 tumors, 30 days after i.v. administration of AduPARE1A (5×10^{10} vp) or saline solution ($n = 6$). B) Quantification of the number of tumorspheres grown as anchorage-independent colonies from CP15 and CP13 saline ($n = 7$) and treated tumors ($n = 6$) respectively 22 days after plating. CP15 tumorsphere representative images (right). Scale bars 100 μm .

population and an increase in tumorsphere number and size. The fact that the *in vivo* effects of AduPARE1A treatment on the CP15 and CP13 PCSC population showed decreased sphere formation but no significant changes in the percentage of CD133⁺ cells may indicate that additional PCSC present in tumorspheres, not detected by CD133 positivity, would be sensitive to AduPARE1A treatment. This highlights the need to investigate for additional PCSC cellular markers as better predictors of AduPARE1A response.

Nevertheless, our data suggest that AduPARE1A might be able to kill PCSC *in vivo*, similarly to what was seen *in vitro* and this can be appealing for pancreatic cancer treatment. Interestingly, a subpopulation of uPAR positive cells has been identified in small cell lung cancer cell lines, derived from lung and from bone marrow and brain metastasis, which possess multi-drug resistance and clonogenic activity (Gutova et al., 2007). uPAR positive cells identified in the small cell lung cancer H466 were capable of forming spheres and efficiently formed transplantable tumors (Qiu et al., 2012). uPAR signaling has also been reported to induce cancer stem cell like properties in breast cancer cells (Jo et al., 2010). Recently, it has been shown that uPAR is important in the maintenance of stem cells and is highly expressed in glioma initiating cells. The regulation of malignant stem-cell renewal was proposed to be through the activation of several components of the hedgehog pathway (Gopinath

et al., 2013). Moreover, a recent study provides evidences of mutual regulation mechanisms among uPAR and beta-catenin signaling in cancer stemness in medulloblastoma (Asuthkar et al., 2012). Although there are no specific data on uPAR signaling in pancreatic cancer stem cells, in the present work we show that tumorspheres from both CP15 and CP13 tumors highly express the uPAR gene, supporting a role of uPAR in pancreatic CSC biology as well as the activity of the uPAR promoter in the PCSC population.

Taken together, these results indicate that uPAR promoter is active in pancreatic cancer stem cells and uPAR-controlled oncolytic adenoviruses, on top of eliminating pancreatic epithelial cancer cells, as we have previously reported (Huch et al., 2009) are able to kill neoplastic cells with PCSC properties *in vitro*. Moreover the antitumor effects of AduPARE1A and the lack of enrichment in PCSC features of treated tumors support the *in vivo* killing of pancreatic cancer stem cells.

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