Pancreatic Transduction by Helper-Dependent Adenoviral Vectors via Intraductal Delivery

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Short title: HDAd-mediated gene transfer to the pancreas

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ABSTRACT

Pancreatic gene transfer could be useful to treat several diseases, such as diabetes mellitus, cystic fibrosis, chronic pancreatitis or pancreatic cancer. Helper-dependent adenoviral vectors (HDAds) are promising tools for gene therapy because of its large cloning capacity, high levels of transgene expression and long-term persistence in immunocompetent animals. Nevertheless, the ability of HDAds to transduce the pancreas *in vivo* has not been investigated yet. Here, we have generated HDAds carrying pancreatic specific expression cassettes i.e. driven either by the elastase or insulin promoter, using a novel and convenient plasmid family and homologous recombination in bacteria. These HDAds were delivered to the pancreas of immunocompetent mice via intra-pancreatic duct injection. HDAds, encoding a CMV-GFP reporter cassette, were able to transduce acinar and islet cells, but transgene expression was lost 15 days post-injection in correlation with severe lymphocytic infiltration. When HDAds encoding GFP under the control of the specific elastase promoter were used, the expression was detected in acinar cells, but similarly, the expression almost disappeared 30 days post-injection and lymphocytic infiltration was also observed. In contrast, long-term transgene expression (>8 months) was achieved with HDAds carrying the insulin promoter and the secretable alkaline phosphatase as reporter gene. Notably, transduction of the liver, the preferred target for Ads, was minimal using this route of delivery. These data indicate that HDAds could be used for pancreatic gene therapy but the selection of the expression cassette is of critical importance to achieve long-term expression of the transgene in this tissue.

INTRODUCTION

Helper dependent adenoviral (HDAd) vectors (also called 'gutless' or high-capacity) are deleted of all viral coding sequences, providing a higher transport capacity (up to 35 kb) and reduced toxicity in animals compared with first-generation adenoviral (FGAd) vectors (Palmer *et al.*, 2005). Currently, the most widely used protocol for amplification of HDAd vectors is based on a loxP/Cre system to remove the packaging region of the helper virus (Parks *et al.*, 1996). This system also involves a 293cell-derived cell line that expresses the *Cre* recombinase, such as the 116cre cells (Palmer *et al.*, 2003), and a helper virus (E1-deleted) that contains two loxP sites flanking the packaging signal (Parks *et al.*, 1996). In contrast, the HDAd vector retains only the packaging signal and inverted terminal repeats (ITRs) of the Ad, with the rest of the genome comprised of the transgene expression cassette and stuffer DNA. The stuffer sequences are important to ensure a minimal size of about 28 kb of the HDAd genome to be packaged into Ad particles (Parks *et al.*, 1997). Several methods have been developed for construction of the HDAd backbone, including cosmid generation (Hillgenberg *et al.*, 2001), homing endonucleases (Shi *et al.*, 2006), homologous recombination (Toietta *et al.*, 2002) and direct cloning (Parks *et al.*, 1996), the latter being the most commonly used. HDAd backbone plasmids are usually very large (> 30 kb) and the cloning strategy is frequently difficult due to the limited availability of unique restriction sites and low efficiency of ligation. Conversely, homologous recombination in bacteria is a very efficient process and has been used to generate both FGAd and HDAd (Chartier *et al.*, 1996; Toietta *et al.*, 2002).

HDAd are attractive vectors for *in vivo* gene therapy, because of their high level of transgene expression and long-term persistence in non-dividing tissues. Adenoviruses possess a preferential tropism for the liver upon systemic injection, that substantially

hamper the transduction of other tissues (Alemany *et al.*, 2000; Ye *et al.*, 2000). Therefore, many gene therapy approaches based on HDAd are directed to the liver (Brunetti-Pierri et al., 2009a; Dimmock et al., 2011). Nonetheless, using alternative routes of delivery it is possible to target a variety of tissues with HDAd, such as the brain (Zou *et al.*, 2001), muscle (Gilbert *et al.*, 2002), lung (Koehler *et al.*, 2006) or the eye (Kreppel *et al.*, 2002).

Pancreatic disorders, such as pancreatic cancer, chronic pancreatitis or diabetes are amenable to be treated by gene therapy, but efficient and persistent pancreatic expression of candidate genes is usually required. Pancreatic transduction has been attempted with FGAd vectors via different routes, but the efficiency and the duration of the transgene expression is very limited in immunocompetent animals (Ayuso *et al.*, 2004; Ayuso *et al.*, 2006; McClane *et al.*, 1997b; McClane *et al.*, 1997c; Wang *et al.*, 2004). Conversely, we and others have demonstrated highly efficient and long-term transduction of beta cells and exocrine pancreas with adeno-associated viral vectors (AAV) (Jimenez *et al.*, 2011; Wang *et al.*, 2006). To the best of our knowledge, HDAd have not been tested for pancreatic transduction.

Here, we describe a novel and convenient plasmid system to generate HDAd vectors (of any size) based on homologous recombination. HDAd vectors generated using this plasmid system were successfully propagated in 116 cells. Next, we performed a detailed characterization of HDAd-mediated gene transfer to the exocrine and endocrine cells of the pancreas *in vivo* after delivery of the vectors via the pancreatic duct. Interestingly, long-term transgene expression in the pancreas was highly dependent on the promoter and the reporter gene used in the expression cassette. Using a pancreatic specific promoter (insulin) and a secretable protein (alkaline phosphatase) the expression was maintained for more than 8 months in immunocompetent mice. Moreover, the intraductal route reduced very significantly the dissemination of HDAd vectors to other organs, like

the liver, lungs or spleen. Thus, our results demonstrate that HDAd vectors are suitable tools for genetic engineering of the pancreas, but particular attention should be paid to the promoter and the transgene to achieve long-term gene expression in this tissue.

MATERIALS AND METHODS

Animals

Two-month-old C56Bl/6 and ICR male mice (Harlan Teklad, Barcelona, Spain) were fed *ad libitum* with a standard diet (Harlan Teklad) and maintained in the SPF-mouse facility at the Center of Animal Biotechnology and Gene Therapy under a 12-h light/dark cycle (lights on at 8:00 AM). The Ethics Committee on Animal and Human Experimentation from the Universitat Autònoma de Barcelona approved all procedures.

Plasmid construction

pSTK plasmids contained the backbone for generating HDAd vectors (Fig. 1A). All plasmids encoded the ampicillin resistance gene to allow selection in bacteria. The ITRs and packaging signal sequences from adenovirus serotype 5 were present in all plasmids and the viral genome can be released from bacterial backbone using *PmeI* restriction sites (Fig. 1A). Stuffer DNA sequences were derived from the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene and genomic DNA from the C346 cosmid. Shuttle plasmids pshuttle EA1 and pshuttle EA2 were derived from p3T (MoBiTec, Göttingen, Germany), which encodes the ampicillin resistance gene. The homologous sequences that mediate recombination with pSTKs plasmids were: (a) left Ad-ITR and packaging signal, and (b) a fragment of 1.7kb of HPRT stuffer DNA in the case of pshuttle EA1 or a fragment of 2.9 Kb of HPRT stuffer DNA in the case of pshuttle EA2, as shown in Fig. 1B. Both shuttle plasmids contain a multiple cloning site for insertion of the desired cloning cassette.

Transgenes used were: (1) enhanced green fluorescent protein (eGFP) driven by the human cytomegalovirus constitutive promoter (CMV) or the rat elastase I (−205/+8) promoter (kindly provided by R. McDonald, University of Texas Southwestern Medical

Center, Dallas, TX, USA) (Kruse *et al.*, 1993;1995; Ornitz *et al.*, 1985); (2) red fluorescent protein (dsRED-monomer, Clontech Laboratories, Mountain View, CA, USA) driven by the CMV and (3) human placental secretable alkaline phosphatase (seAP) driven by the human insulin promoter (hINS). The human placental seAP cDNA has been used previously by our group for *in vivo* experiments using adeno-associated vectors (Jimenez *et al.*, 2013).

Additional details of the cloning procedures are available upon request.

Homologous recombination in bacteria

The shuttle plasmids pEA1 or pEA2 containing the expression cassette of interest were digested with a restriction enzyme of choice to linearize the plasmid (i.e. cutting the plasmid outside of the homologous recombination region as shown in Fig. 1C. pSTK plasmids were linearized by *EcoRV* overnight digestion. Plasmid DNA was purified by Phenol-chloroform standard procedure after enzymatic digestion. 5 ng of linearized pSTK plasmid was co-transformed (by electroporation) at different molecular ratios (1:1, 1:5, 1:10) with linearized pshuttle plasmid into *E. coli BJ5183* (Stratagene-Agilent technologies, Santa Clara, CA, USA). Recombinant plasmids were screened by restriction enzyme digestion and subsequently transformed in *E. coli XL-2 blue* (Stratagene) to avoid further recombinations/re-arrangements.

Generation of HDAd vectors

To rescue and amplify HDAd vectors, the pFK7, pEA4, pEA9 and pEA11 plasmids (Fig. 2A) were digested with *PmeI* in order to release the viral genome from the bacterial backbone. Subsequently, the viral genome was transfected into 116cre cells and amplified in the presence of E1-deleted helper virus (HV) (F. Kreppel, unpublished data) following

the protocol described by Ng and colleagues (Palmer *et al.*, 2003) (cre116 cells were kindly provided by P. Ng, Baylor college of Medicine, Houston, USA). Briefly, a 6cm culture dish of 116cre cells was transfected with the viral genome (passage 0 or P0) and 48h after HV infection the cells were harvested, viruses were released by freeze/thawing and used to infect a second 6cm plate of 116cre cells in the presence of HV (P1). P1 lysate was used to infect a third 6cm plate (P2), and the P2 lysate was used to infect a 15cm plate (P3). Finally, the P3 lysate was distributed into 20x15cm plates (P4), cells were harvested 48h later, lysed and HDAd vectors were purified by two cesium chloride gradients (one discontinuous and one continuous gradient). Subsequently, desalting PD-10 chromatography columns (GE healthcare life sciences, Little Chalfont, Buckinghamshire, UK) were used. Vectors were formulated in sterile PBS containing 10% glycerol and stored at -80ºC until use. Titration of HDAd particles and determination of % of residual helper virus (HV) was performed by qPCR. Specific primers to amplify the human HPRT region were used to titer HDAd particles (HPRT Forward Primer: 5'-GGGCATAAAGGGTTTTAATGG-3'; HPRT Reverse Primer: 5'-AACTATCAACCTCATCCTCTCCA-3') and specific primers to detect the adenovirus *Fiber* gene were used to quantify helper viruses (Fiber Forward Primer: 5'ATGAAGCGCGCAAGACCGTCT-3'; Fiber Reverse Primer: 5'-TGAGCGCAAGCATGCCATTGG-3'). Titers of HDAd stocks used in this work were: $3x10^{11}$ vp/ml HDAd-CMV-GFP (1.5% HV), $4,8x10^{11}$ vp/ml HDAd-ela-GFP (5.8% HV) and $2,7x10^{11}$ vp/ml of HDAd-hINS-seAP (1.6% HV).

Cell culture and transfection

The acinar cell line 266-6 was obtained from ATCC (Catalog No. CRL-2151) and was cultured with DMEM (4.5 g/l glucose) supplemented with 10% FBS. Flasks and plates

were treated with pig skin gelatine 0.1% for 15 minutes prior to use to avoid clumping. The cell line HEK-293 (Qbiogene-MP biomedicals, Santa Ana, CA, USA) was cultured with DMEM (4.5g/l glucose) supplemented with 10% FBS. The beta-cell line MIN6-B1 (Miyazaki *et al.*, 1990) (kindly provided by J. Miyazaki, Osaka, Japan) was cultured with DMEM (4.5 g/l glucose) supplemented with 15% FBS, 71 μM 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100μg/ml).

Cells were transfected in 24 well-plates with X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland).

In vivo administration of HDAd vectors

Mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The duodenum was isolated with the common bile duct attached. A microclamp was placed on the bile duct caudal to the liver and a 30 gauge needle was used to advance retrogradely through the sphincter of Oddi into the common bile duct. The needle was secured in place with a second microclamp around the bile duct and 100 μl of HDAd solution (containing $2x10^{10}$vp diluted in PBS) was injected into the duct over approximately 1 min. At 1 min post-injection the microclamps and needle were removed. For systemic administration, 200 μl HDAd was injected via the jugular vein.

Immunohistochemical analysis

Tissue samples were fixed in 10% formalin, embedded in paraffin and sectioned. Double GFP and insulin immunostaining was performed with goat anti-GFP (Abcam, Cambridge, MA, USA) and guinea pig anti-insulin (Sigma-Aldrich, St Louis, MO, USA) antibodies. As secondary antibodies, biotinylated donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TRITC-conjugated goat anti-guinea pig (Life

Technologies, Carlsbad, CA, USA) were used. Streptavidine Alexa Fluor 488 (Life Technologies) and 3,3-Diaminobenzidine (DAB) were used for fluorescence imaging and bright field, respectively. Sections were counterstained with Hoechst (Sigma-Aldrich) for nuclear counterstaining of fluorescent specimens. Haematoxylin and Eosin staining was used for the bright field images.

Quantification of transduction

Detection of GFP was performed in three pancreas sections per animal (3-4 animals/group; sections 200 μm apart). Quantification of transduction of the exocrine pancreas was performed in ten random fields per section. The transduced exocrine area and total area of each field were determined by image analysis with a microscope (Eclipse E800; Nikon, Tokyo, Japan) connected to a video camera and an image analyzer (analySIS 3.0; Soft Imaging System, Center Valley, PA, USA). The percentage of transduced exocrine pancreas was calculated by dividing the transduced exocrine area by the total exocrine area. The percentage of transduced beta cells per islet was calculated by dividing the number of all double insulin$^+$ and GFP$^+$ cells in one islet by the number of all insulin$^+$ of that islet.

Vector genome copy number

Vector genome copy number was measured from frozen or paraffin-embedded tissues. DNA was isolated from paraffin blocks containing pancreas tissue using the NucleoSpin® FFPE DNA kit (Macherey-Nagel, Düren, Germany) according to kit specifications. Briefly, seven sections of 10 □m thickness were cut for each animal sample and paraffin dissolved. After paraffin dissolution, tissue was digested o/n by proteinase K in lysis buffer. DNA was then decrosslinked, precipitated by ethanol and purified in the kit

columns. Total DNA from frozen tissues was isolated with a MasterPureDNA Purification Kit (Epicentre, Madison, WI, USA) after o/n digestions in proteinase K (0.2 mg/ml). Resulting DNA from paraffin-embedded or frozen tissues were diluted in distilled water to 100 ng DNA/□l. The vector genome copy number in 100 ng of total DNA was determined by qPCR with primers and probe specific for the HPRT stuffer DNA sequence that is incorporated in all of the HDAd vectors used in this study (see Figure 2); Forward primer: 5′-CTGGTCTGCAGTTAGGAGATAC-3′; reverse primer: 5′-CAGAGGCTCAGAGAGGTTAAAT-3′; probe: 5′-ACTTCTTAGCCGTGTCACCTTGGG-3′.

The final values were determined by comparing to a reference standard curve, built from serial dilutions of the linearized plasmids used for HDAd vector production spiked into 100 ng of non-transduced genomic DNA.

Measurement of serum alkaline phosphatase levels

Circulating seAP levels were determined from serum samples with placental seAP Tropix PhosphaLight chemiluminescent reporter gene assay (Life Technologies). Human placental seAP can be discriminated from endogenous seAP in mice by incubating serum samples for 30 min at 65°C. This pre-treatment ensures inactivation of endogenous SeAP but not the transgene-derived placental seAP, since the latter is a thermoresistant enzyme, as already shown in the serum of animals injected with AAV vectors expressing this transgene (Jimenez *et al.*, 2013).

Statistical analysis

All values are expressed as means ± SEM. Differences between groups were compared by unpaired Student's t test. A **p* value < 0.05 and ***p* value <0.01 was

considered statistically significant.

RESULTS

Novel plasmid family to generate HDAd by homologous recombination

Helper-dependent adenoviral genome should have a final size above 28 Kb to allow efficient packaging in the Ad capsid (Parks *et al.*, 1997). To generate these vectors, the HDAd backbone maintains the viral ITRs and the packaging signal while all coding adenoviral genes are deleted. Here, we have generated a novel HDAd backbone plasmid family (named pSTKs) that contains the Ad ITRs and packaging signal with different fragments of stuffer DNA to allow the accommodation of expression cassettes of any size. Several fragments (8.8 kb, 16 kb, 19 kb or 20 kb) of human DNA from the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus were used as stuffer DNA in all plasmids, except for the pSTK42 (Fig. 1A). Fragments of genomic DNA from the C346 cosmid were also used in pSTK129, pSTK120 and pSTK119 plasmids (Fig. 1A). All pSTKs plasmids were constructed using pBluescript (Agilent technologies) as bacterial backbone and *PmeI* restriction sites were located at both ends of the ITRs to easily remove the viral genome from the bacterial sequences. The cloning of expression cassettes by direct ligation into pSTKs plasmids is difficult because of the large size and the limited availability of unique restriction sites. Conversely, it has been shown that homologous recombination in bacteria is a more efficient method compared with direct ligation to generate both adenovirus (Chartier *et al.*, 1996) and also HDAd vectors (Toietta *et al.*, 2002). Here, we generated two different shuttle plasmids to be used for homologous recombination with pSTK plasmids, namely pshuttle EA1 and pshuttle EA2 (Fig. 1B). The left Ad ITR and the packaging signal was excised by *PmeI-EcoRV* digestion from the pSTK129 and cloned into the *ApaI* site of the p3T plasmid, being this fragment the left arm

for homologous recombination. Next, pSTK129 was digested by *EcoRV-FspAI* and a fragment of 1.7 kb belonging to the HPRT stuffer DNA (depicted in grey in Fig. 1A and Fig. 1B) was released and cloned into the EcoICRI site of p3T, thus representing the right arm for homologous recombination. pshuttle EA2 had the same left arm for recombination, but a different fragment of HPRT stuffer DNA (shown as a black box in Fig. 1A and Fig. 1B) obtained by digestion with *EcoRV* and *PvuII* as the right arm for recombination. This fragment was also cloned into the EcoICRI site of p3T. In both shuttle plasmids the multicloning site (MCS) was maintained to facilitate the cloning of the desired expression cassette. As shown in Fig. 1A and 1B, the pShuttle EA1 was designed for homologous recombination with pSTK129 and pSTK134 and the pShuttle EA2 for recombination with pSTK120, pSTK119, pSTK68 and pSTK51.

Amplification of HDAd vectors generated by homologous recombination

For homologous recombination in bacteria, the shuttle plasmid containing the transgene of interest was linearized by a restriction enzyme cutting the bacterial backbone and the pSTK plasmid was digested by *EcoRV* (located in between of the packaging signal and the stuffer DNA in all pSTK plasmids). Both linearized plasmids were co-transformed into *E.coli BJ5183* to facilitate homologous recombination as shown in Fig. 1C.

Using this method we have generated three novel HDAd genomes (pEA4, pEA9 and pEA11) and have compared the propagation and rescue of these vectors with a HDAd plasmid generated by standard ligation (pFK7) (Fig. 2). HDAd-CMV-RFP (named pEA4) expressed the red fluorescent protein (RFP) under the control of the cytomegalovirus ubiquitous promoter (CMV), HDAd-ela-GFP (pEA9) expressed the green fluorecent protein (GFP) under the control of the pancreatic specific elastase promoter (Kruse *et al.*,

1993;1995) and HDAd-hINS-seAP (pEA11) expressed the human placental derived secretable alkaline phosphatase under the control of the human insulin promoter (Fig. 2A). To generate pEA4, the expression cassette (CMV-RFP) was cloned into the pshuttle EA1 and inserted into pSTK129 by homologous recombination as shown in Fig. 1C. The same approach was used to generate pEA9. pEA11 was constructed by cloning the expression cassette (hINS-seAP) into the pshuttle EA2 and the homologous recombination was performed with the pSTK119.

As shown in Fig. 2B, there was an increase in the number of GFP and RFP cells from P1 to P4 indicating a correct amplification of HDAd vectors generated by homologous recombination. Surprisingly, GFP was expressed in 116cre cells under the control of the elastase promoter, a highly specific pancreatic promoter in transgenic mice (Ornitz *et al.*, 1985) and animals transduced with viral vectors *in vivo* (Jimenez *et al.*, 2011). Similarly, the insulin promoter was also active in 116cre as evidenced by an increase in seAP levels in culture media of P3 and P4 amplifications (Fig. 2C). To further investigate the specificity of these pancreatic promoters in cell lines in culture we transfected CMV-seAP, elastase-seAP or insulin-seAP constructs in HEK-293, 266-6 (mouse acinar cells) and MIN6-B1 cells (mouse beta cells) (Fig. 2D, 2E, 2F). Our data suggested that HEK-293 and 116cre cells are quite promiscuous regarding promoter specificity. In contrast, the elastase promoter was active in 266-6 cells but not in MIN6-B1 cells whereas the insulin promoter was active in MIN6-B1 but not in 266-6 cells.

Characterization of pancreatic tranduction mediated by HDAd expressing GFP

The efficiency of FGAd vectors to transduce the pancreas via different routes has been investigated in the past (Ayuso *et al.*, 2004; McClane *et al.*, 1997a; McClane *et al.*,

1997b; McClane *et al.*, 1997c; Wang *et al.*, 2004). However, to date no studies have been performed with HDAd vectors. The capsid proteins dictate the tropism of HDAd and FGAd vectors, thus the transduction pattern in tissues should be similar in both cases. However, FGAds are highly immunogenic *in vivo* and transduced cells are quickly eliminated in immunocompetent animals (Liu *et al.*, 2003). In contrast, HDAds have demonstrated long-term persistence in several tissue in small and large animal models (Brunetti-Pierri *et al.*, 2009a). Here, we investigated the efficiency of transduction and the duration of transgene expression mediated by HDAd in the pancreas.

First, a dose of $2x10^{10}$ vp of HDAd-CMV-GFP vectors was injected via the pancreatic duct into immunocompetent ICR mice. These mice were sacrificed 5, 15 or 30 days after vector delivery and the expression of GFP was analyzed by immunohistochemistry. Widespread pancreatic GFP expression was detected five days post-injection (PI) (Fig. 3A) and about 3% of total acinar cells were transduced (Fig. 3B). The percentage of GFP-expressing cells was reduced drastically 15 days PI and virtually disappeared at 30 days PI (Fig. 3A and Fig. 3B). Using a double immunostaining against GFP and insulin, quantification of the percentage of β-cells transduced by this vector and this delivery route was performed. About 1% of insulin-positive cells were transduced at day 5 PI (with 13.6% of islets having at least one GFP-positive cell), but these GFP^+ cells decreased in a similar fashion than in acinar cells (Fig. 3C and 3D) with no expression detected at 15 and 30 days PI. Notably, we found significant lymphocytic infiltration in the pancreas of mice sacrificed 5 days PI, being almost undetectable at 15 and 30 days PI (Fig. 3A). Lymphocytes were also present surrounding the pancreatic islets expressing GFP (Fig. 3D).

Next, a dose of $2x10^{10}$ vp of HDAd-ela-GFP vectors was injected via the pancreatic duct into immunocompetent ICR mice. Animals were sacrificed and analyzed as described

above. Widespread transduction of the pancreas at 5 days PI was found, being the acinar transduction about 1.5% (Fig. 4A and 4B). Although GFP^+ cells were significantly reduced both at 15 and 30 days PI, positive cells were still maintained 30 days PI (Fig. 4A and 4B). Using the elastase promoter we found only one islet that contained GFP/insulin double positive cells (Fig. 4C), which is consistent with the low activity of this promoter in endocrine cells (Jimenez *et al.*, 2011; Ornitz *et al.*, 1985). Lymphocytic infiltration was mainly detected in the pancreas of mice 15 days PI but also some lymphocytes were present at 30 days PI, whereas minimal infiltration was detected 5 days PI (Fig. 4A).

Non-invasive monitoring of cell transduction and long-term persistence of HDAd vectors in the pancreas

Secretable alkaline phosphatase (seAP) has been used as a marker transgene in several studies mainly because it is secreted into the blood very efficiently (Bartoli *et al.*, 2006; Jimenez *et al.*, 2013; Riviere *et al.*, 2006; Shiraiwa *et al.*, 2007). In addition, the placental-derived seAP is a thermoresistant enzyme that can be easily differentiated from the endogenous alkaline phosphatase. We hypothesized that using HDAd-seAP vectors it could be possible to monitor long-term pancreatic transduction in living mice. To this end, HDAd vectors encoding the placental-derived seAP gene under a specific pancreatic promoter, i.e. the human insulin promoter (hINS), were generated (Fig. 2). A dose of $2x10^{10}$vp of HDAd-hINS-seAP vectors was injected via the pancreatic duct into immunocompetent C57Bl/6 mice (Fig. 5A) and ICR mice (Fig. 5B). We tested the vectors in both strains because it has been demonstrated that immunological responses against adenoviral vectors could be different depending on the mouse strain (Rawle *et al.*, 2004).

SeAP was detected in the serum of both strains of mice at similar levels and the expression was maintained up to 8 months PI. On the other hand, seAP was non detected in

the serum of mice treated with the same vector dose but delivered intravenously (Fig. 5A and 5B); demonstrating that (i) Ads do not reach the β-cells via intravenous delivery, as previously observed (Ayuso *et al.*, 2004), and (ii) the insulin promoter is not active in non-β cells.

In agreement with the long-term expression, we did not observe any sign of inflammation in the pancreas of C57Bl/6 mice (Fig. 5C) and ICR mice (Fig. 5D) sacrificed at this time point. These data demonstrated that HDAd vectors were not immunogenic when injected into the pancreas if an appropriate combination of promoter and reporter gene is used.

Reduced systemic dissemination of HDAd vectors by intraductal administration

One of the major limitations of HDAd vectors for gene therapy is the toxicity triggered by systemic diffusion of these vectors, that can be lethal at high doses (Brunetti-Pierri *et al.*, 2004). Local administration of HDAd vectors has been investigated for liver-directed gene therapy resulting in increased therapeutic index of the therapy, i.e. better transduction of the target organ with reduced systemic dissemination (Brunetti-Pierri et al., 2012; Brunetti-Pierri et al., 2009b).

Here, we investigated the systemic dissemination of HDAd-hINS-seAP vectors administered via intraductal delivery (ID) in comparison with the intravenous (IV) injection. As expected, upon IV administration of HDAd vectors most of the vector genomes were found in the liver, lungs and spleen (Fig. 6A and (Brunetti-Pierri et al., 2005b)). Importantly, we detected a highly significant reduction of systemic dissemination of HDAd to these organs when the same dose of vectors was administered ID (Fig. 6A). In

agreement, no GFP^+ cells were detected in the liver of both HDAd-CMV-GFP and HDAd-ela-GFP treated animals (supplementary Fig. 1).

The quantity of vector genomes found in the pancreas was very low both in the ID and IV injected animals, although more vector genomes were found in the animals injected ID (Fig. 6A and 6B). Despite the low vector copy number found in the pancreas, efficient transduction of this tissue was achieved in the ID group but not in the IV group (Fig. 6C, Fig. 5A and 5B).

We also investigated the persistence of HDAd vector genomes at several time points post-infection in the pancreas of the animals injected ID with HDAd-CMV-GFP, HDAd-ela-GFP and HDAd-hINS-seAP. HDAd vector genomes showed a tendency to decrease 30 days after vector administration in HDAd-CMV-GFP mice and to a less extend in HDAd-ela-GFP mice (Supplementary Fig. 2A and 2B). Persistent vector genomes were detected in the pancreas 8 months after ID administration of HdAd-hINS-seAP vectors (Supplementary Fig. 2C).

Altogether, our data demonstrate that intraductal delivery resulted in significantly higher efficient pancreatic transduction with reduced systemic vector dissemination compared with the systemic intravascular delivery of HDAd vectors.

DISCUSSION

Promising results have been obtained for gene therapy of hemophilia B (Brunetti-Pierri *et al.*, 2005a) , hyperbilirrubinemia (Dimmock *et al.*, 2011), glycogen storage disease type Ia (Crane *et al.*, 2012) and glioblastoma (Muhammad *et al.*, 2013) using helper-dependent adenoviral vectors targeting the liver and the brain. In the current work we investigated the utility of HDAd vectors for pancreatic gene transfer. Our group has previously shown that FGAd vectors are able to transduce the pancreas when the hepatic circulation was blocked in mice (Ayuso *et al.*, 2004) or when the vectors were delivered into clamped pancreatic vessels of dogs (Ayuso *et al.*, 2006). Noteworthy, adenoviruses can also be retrogradelly injected into the common bile duct to transduce the pancreas. Using this loco-regional route, efficient and selective transduction of the pancreas was achieved using FGAd and also oncolytic Ad vectors (Jose *et al.*, 2013). Antitumoral responses were stronger by ductal viral application than intravenously, in line with the 38-fold increase in pancreas transduction observed upon ductal administration (Jose *et al.*, 2013). Similarly, adeno-associated viral vectors (AAV) transduced the pancreas highly efficiently and persistently after delivery via the pancreatic duct (Jimenez *et al.*, 2011; Wang *et al.*, 2006). Altogether these data prompted us to investigate pancreatic gene transfer with HDAd vectors by this local route of delivery. Using HDAd-CMV-GFP vectors, about 3% of exocrine cells and 1% of endocrine cells were GFP-positive, being this efficiency significantly lower compared with that of AAV vectors (Jimenez *et al.*, 2011). One way of increasing this efficiency could be to increase the vector dose, as we observed previously with AAV (Jimenez *et al.*, 2011) and production of highly concentrated HDAd vector stocks is possible using novel technologies (Palmer *et al.*, 2008). However, a potential problem of using high doses of HDAd is the innate immune response and the acute toxicity that could lead to death (Brunetti-Pierri *et al.*, 2004); this

type of toxicity is mostly due to liver and spleen transduction in particular due to scavenging by macrophages and their activation. Importantly, here we have shown a significant reduction in systemic HDAd vector dissemination upon intraductal delivery with minimal detection of vector genomes in the liver, lungs and spleen compared with intravenous route. Consistently, no expression of GFP was detected in the liver upon administration of HDAd-CMV-GFP via intraductal delivery. This is a major advantage of the intraductal route of administration compared with the pancreatic transduction mediated by intravenous delivery of HDAd in mice with clamped circulation, since the latter route resulted in strong expression of the marker gene in hepatocytes (supplementary Fig. 3) unless a pancreatic specific promoter is used (supplementary Fig. 4).

One of the most desired properties of HDAd vectors is the ability to mediate long-term transgene expression in immunocompetent animals. However, the expression of GFP in the pancreas of HDAd-CMV-GFP-treated mice lasted only a few days and correlated with abundant lymphocytic infiltration. This infiltration was resolved 30 days post-injection when transgene expression had been lost. At this time point and also 60 and 90 days post-injection, the pancreatic function was not altered as documented by normal serum levels of biochemical markers of pancreatic damage, such as amylase and lipase (data not shown). Altogether, these data indicated that lymphocytic infiltration was selective for the transgene (GFP), probably because of its expression in antigen presenting cells mediated by the ubiquitous CMV promoter. Similarly, expression of β-Gal in the skeletal muscle of immunocompetent mice, using HDAd vectors, resulted in a decrease in vector copy number and complete loss of β-Gal expression in mice (Chen *et al.*, 1997). These mice showed extensive inflammatory cellular infiltration consisting primarily of CD4 and CD8 lymphocytes, associated with the expression of β-Gal (Chen *et al.*, 1997). Loss of expression was also observed when using HDAd β-Gal vectors in lung (Toietta *et*

al., 2003). Weaver and colleagues clearly demonstrated that HDAd are able to elicit significant transgene-directed T cell and antibody responses (Weaver *et al.*, 2009); whereas Kron et al. have shown that HDAd vectors efficiently induced multispecific transgene product-directed CD8 cytotoxic T lymphocytes (Kron *et al.*, 2011). Furthermore, it has been shown that interferon-γ, which would be produced in the course of T cell-mediated immune response, can inhibit the CMV promoter (Harms *et al.*, 1995). Although immune-mediated repression of the CMV promoter cannot be completely ruled out, our data showing a reduction in the vector genomes in the pancreas 30 days post-infection in animals injected with HDAd-CMV-GFP suggested that immune-mediated destruction of transduced cells was the most likely explanation for transgene lost. In HDAd-ela-GFP injected animals, vector genome reduction in the pancreas 30 days post-infection was less pronounced, which is in agreement with a more tardive lymphocytic infiltration of the pancreas of these animals compared with HDAd-CMV-GFP injected animals.

Importantly, using a mammalian gene such as human seAP we did not observe loss of the transgene expression and we detect HDAd vector genomes in the pancreas of the animals 8 months post administration, thus indicating a minimal (if any) immune response against this protein in our experimental model. The high protein homology of human seAP and murine seAP (more than 77% according to BLAST) could also account to explain the lack of immunogenicity. Moreover, the fact that seAP expression was limited to β-cells, because of the insulin promoter, could also prevent activation of antigen presenting cells and transgene-directed T cell responses. In agreement, the use of a liver specific promoter has been shown to reduce immune responses against the transgene product in the context of Ad vectors (Pastore *et al.*, 1999).

We would also like to highlight the sensitivity of the seAP as a reporter transgene. Only 1% of beta cells transduced by HDAds were sufficient to produce detectable seAP

levels in serum. seAP is also convenient because it can be measured longitudinally (i.e. different time points) in living mice.

An additional value of the present work is the novel plasmid family developed to generate HDAd genomes. We proved that both shuttle plasmids constructed here (pshuttleEA1 and pshuttleEA2) mediated homologous recombination with the HDAd backbone (pSTK) at the expected sites. Moreover, HDAd vectors generated with this system propagated normally in cre116 cells and the vectors were infective *in vivo* validating this technology.

In summary, we have shown that HDAd vectors transduce the acinar and endocrine pancreas *in vivo* via pancreatic duct delivery. These data suggest that pancreatic gene transfer/therapy approaches for the treatment of diabetes mellitus, chronic pancreatitis or pancreatic cancer may benefit from HDAd vectors as delivery tools.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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FIGURE LEGENDS

FIG. 1. Schematic representation of the novel plasmid family to generate HDAd vectors by homologous recombination. (A) Scheme of the HDAd vectors backbones used in this study. In all cases the vector backbone is released from the bacterial plasmid using *PmeI* sites located at both ends of the ITRs. The name of the plasmid and the cloning capacity is shown. (**B**) Scheme of the p3T plasmid, highlighting the restriction enzymes used for the generation of pShuttle EA1 and pShuttle EA2. Unique restriction sites maintained in the multicloning site are depicted in pShuttle EA1 and pShuttle EA2. (**C**) Simulation of the homologous recombination that should take place in *E.coli BJ5183* bacteria after transformation with linearized pShuttle plasmid and the pSTK plasmid.

FIG. 2. Amplification of HDAd vectors generated by homologous recombination. (A) Scheme of HDAd genomes. pEA4, pEA9 and pEA11 were generated by homologous recombination and pFK7 was generated by direct cloning. (**B**) Serial amplifications of HDAd vectors in cre116 cell line. P0 is the transfection of 116 cells with the HDAd backbone; P1, P2, P3 and P4, are subsequent viral amplifications (1^{st} to 4^{th}, respectively) Representative fluorescent images are shown for HDAd-CMV-GFP, HDAd-CMV-RFP and HDAd-ela-GFP. Bright field images are shown for HDAd-hINS-seAP. Original magnification 100X. (**C**) Quantification of seAP in culture media of 116cre cells at each amplification step. (**D**) Quantification of seAP in culture media of HEK-293 cells transfected with CMV-seAP, elastase-seAP, insulin-seAP or null (irrelevant) plasmids. (**E**) Quantification of seAP in culture media of 266-6 murine acinar cells. (**F**) Quantification of seAP in culture media of MIN6-B1 murine beta cells transfected.

FIG. 3. Pancreatic transduction by HDAd-CMV-GFP delivered via intraductal delivery. ICR mice were injected intraductally with 2×10^{10} vp of HDAd encoding the GFP reporter gene driven by the CMV ubiquitous promoter. Animals were analyzed 5, 15 and 30 days after injection. (**A**) Immunohistochemical analysis of GFP (green, top panel) and Hematoxilin/Eosin staining of pancreatic sections (bottom panel). Arrows point to the lymphocytic infiltration. (**B**) Quantification of GFP-positive exocrine cells per pancreas (%). (**C**) Quantification of GFP-positive beta cells (double positive for GFP and insulin, as shown in D) per pancreas (%). (**D**) Immunohistochemical analysis of GFP (green) and insulin (red). Arrow pointed at double positive cells. Results are expressed as mean ± SEM; n = 4-6 animals per group. *p < 0.05 *vs* day 5. ND, not detected. Original magnification (**A)** ×200 and (**D**) ×100.

FIG. 4. Pancreatic transduction by HDAd-ela-GFP delivered via intraductal delivery. ICR mice were injected intraductally with 2×10^{10} vp of HDAd encoding the GFP reporter gene driven by the pancreatic specific elastase promoter. Animals were analyzed 5, 15 and 30 days after injection. (**A**) Immunohistochemical analysis of GFP (green, top panel) and Hematoxilin/Eosin staining of pancreatic sections (bottom panel). Arrows point to the lymphocytic infiltration. (**B**) Quantification of GFP-positive exocrine cells per pancreas (%). (**C**) Immunohistochemical analysis of GFP (green) and insulin (red). Arrow point at a double positive cell. Results are expressed as mean ± SEM; n = 4-6 animals per group. **p < 0.01 *vs* day 5. ND, not detected. Original magnification (**A**) ×200 and (**C**) ×400.

FIG. 5. Long-term expression of seAP in immunocompetent mice after HDAd-hINS-seAP intraductal delivery. C56Bl/6 **(A)** and ICR **(B)** mice were injected intraductally or intravenously with 2×10^{10} vp of HDAd encoding the seAP reporter gene driven by the pancreatic specific insulin promoter. seAP levels were monitored periodically in serum samples of these mice as indicated in Material and Methods. Hematoxilin/Eosin staining of pancreatic (top panel) and liver (bottom panel) sections of C56Bl/6 mice (**C**) and ICR mice (**D**) 8 months after HDAd-hINS-seAP delivery. ID, intraductal; IV, intravenous; Con, control healthy mice (n=9-10 per group). Original magnification ×100. * $p<0.05$ vs IV, ** $p<0.01$ vs IV.

FIG 6. Vector genome biodistribution in ICR mice after intraductal or intravenous administration of HDAd. A dose of $2x10^{10}$ vp of HDAd-hINS-seAP were administered by intraductal injection (ID) or intravenously (IV) to ICR mice. Two weeks post-injection the vector genomes were quantified by qPCR in several tissues **(A, B)** and seAP levels was measured in the serum **(C)**. Values shown are means ± SEM (n= 4-5 animals per group). * $p<0.05$ vs ID, ** $p<0.01$ vs ID.

Supplementary FIG. 1. Transgene expression in the liver. Immunohistochemical analysis of GFP (green, top panel) and Hematoxilin/Eosin staining (bottom panel) of liver sections 5 days post HDAd vector injection (n=4-5 per group). Con, control healthy mice. Original magnification ×100.

Supplementary FIG. 2. Vector genome quantification from paraffin embedded pancreatic samples. DNA was extracted from pancreatic tissue embedded in paraffin as

described in Material and Methods and HDAd genome quantification was performed by qPCR. **(A)** Vector genome quantification in the pancreas of ICR mice 5 and 30 days after HDAd-CMV-GFP vector administration (n=4-5 animals per group). **(B)** Vector genome quantification in the pancreas of ICR mice 5 and 30 days after HDAd-ela-GFP vector administration (n=4-5 animals per group). **(C)** Vector genome quantification in the pancreas of ICR mice 8 month after HDAd-hINS-seAP vector administration via intraductal (ID) or intravenous (IV) (n=9-10 animals per group).

Supplementary FIG. 3. Transgene expression in pancreatic islets and liver after systemic deliver of HDAd vectors in mice with clamped hepatic circulation. ICR mice were injected intravenously with 8×10^{10} vp of HDAd encoding the GFP reporter gene driven by the CMV ubiquitous promoter in mice with blocked hepatic circulation as described in (Ayuso *et al.*, 2004). Animals were analyzed 5 days after injection (n=5 per group). Arrows pointed to the lymphocytic infiltration. Immunohistochemical analysis of GFP (brown) in pancreas (**A,B,C,D**) and liver (**E,F**). Original magnification (**A,B,C,D,F**) ×200 and (**E**) ×100.

Supplementary FIG. 4. Restricted expression of the transgene in beta cells using adenoviral vectors and the insulin promoter. ICR mice with clamped hepatic circulation were injected intravenously with 8×10^{10} vp of FGAd encoding the GFP reporter gene driven by the pancreatic specific insulin promoter. Animals were analyzed 5 days after injection (n=5 per group). Immunohistochemical analysis of GFP (brown) in pancreas (**A,B,C**) and liver (**D**). Arrows point to transduced pancreatic islets. Original magnification (**A,B,D**) ×200 and (**C**) ×40.