Pancreatic Transduction by Helper-Dependent Adenoviral Vectors via Intraductal Delivery

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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 (HDAd) are promising tools for gene therapy because of their large cloning capacity, high levels of transgene expression, and long-term persistence in immunocompetent animals. Nevertheless, the ability of HDAd to transduce the pancreas *in vivo* has not been investigated yet. Here, we have generated HDAd carrying pancreas-specific expression cassettes, that is, driven either by the elastase or insulin promoter, using a novel and convenient plasmid family and homologous recombination in bacteria. These HDAd were delivered to the pancreas of immunocompetent mice via intrapancreatic duct injection. HDAd, encoding a CMV-GFP reporter cassette, were able to transduce acinar and islet cells, but transgene expression was lost 15 days postinjection in correlation with severe lymphocytic infiltration. When HDAd encoding GFP under the control of the specific elastase promoter were used, 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 HDAd carrying the insulin promoter and the secretable alkaline phosphatase as the reporter gene. Notably, transduction of the liver, the preferred target for adenovirus, was minimal by this route of delivery. These data indicate that HDAd could be used for pancreatic gene therapy but that 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 vectors) 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 and Ng, 2005). At present, 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 293 cell-derived cell line that expresses the Cre recombinase, such as 116cre cells (Palmer and Ng, 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 adenovirus, with the rest of the genome composed 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 adenoviral particles (Parks and Graham, 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 large (>30 kb) and the cloning strategy is frequently difficult because of the limited availability of unique restriction sites and low efficiency of ligation. Conversely, homologous

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recombination in bacteria is an efficient process and has been used to generate both FGAd and HDAd (Chartier *et al.*, 1996; Toietta *et al.*, 2002).

HDAds are attractive vectors for *in vivo* gene therapy, because of their high level of transgene expression and long-term persistence in nondividing tissues. Adenoviruses possess a preferential tropism for the liver on systemic injection, which substantially hampers 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 and Ng, 2009; Dimmock *et al.*, 2011). Nonetheless, by 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), and eye (Kreppel *et al.*, 2002).

Pancreatic disorders, such as pancreatic cancer, chronic pancreatitis, and diabetes, are amenable to treatment by gene therapy, but efficient and persistent pancreatic expression of candidate genes is usually required. Pancreatic transduction has been attempted with FGAd vectors via various routes, but the efficiency and duration of transgene expression is limited in immunocompetent animals (McClane *et al.*, 1997b, c; Ayuso *et al.*, 2004, 2006; 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 (AAV) vectors (Wang *et al.*, 2006; Jimenez *et al.*, 2011). To the best of our knowledge, HDAds 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 with this plasmid system were successfully propagated in 116cre 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 pancreas-specific promoter (insulin) and a secretable protein (alkaline phosphatase), expression was maintained for more than 8 months in immunocompetent mice. Moreover, the intraductal route reduced significantly the dissemination of HDAd vectors to other organs, such as the liver, lungs, and 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 C57BL/6 and ICR male mice (Harlan Teklad, Barcelona, Spain) were fed *ad libitum* with a standard diet (Harlan Teklad) and maintained in the specific pathogen-free (SPF) mouse facility at the Center of Animal Biotechnology and Gene Therapy under a 12-hr light-to-dark cycle (lights on at 8:00 A.M.). The Ethics Committee on Animal and Human Experimentation from the Universitat Autònoma de Barcelona (Bellaterra, Spain) approved all procedures.

Plasmid construction

pSTK plasmids contain 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 could be released from the bacterial backbone by using *PmeI* restriction sites (Fig. 1A). Stuffer DNA sequences were derived from the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene and genomic DNA was 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 pSTK plasmids were as follows: (1) left Ad-ITR and packaging signal, and (2) a 1.7-kb fragment of HPRT stuffer DNA in the case of pShuttle EA1 or a 2.9-kb fragment 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 as follows: (1) enhanced green fluorescent protein (eGFP) driven by the human cytomegalovirus (CMV) constitutive promoter or the rat elastase I (−205/+8) promoter (kindly provided by R. MacDonald, University of Texas Southwestern Medical Center, Dallas, TX) (Ornitz *et al.*, 1985; Kruse *et al.*, 1993, 1995); (2) red fluorescent protein (dsRED-monomer; Clontech Laboratories, Mountain View, CA) driven by the CMV promoter; and (3) human placental secretable alkaline phosphatase (seAP) driven by the human insulin (hINS) promoter. 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 on request.

Homologous recombination in bacteria

Shuttle plasmids pEA1 and pEA2 containing the expression cassette of interest were digested with the 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 overnight *EcoRV* digestion. Plasmid DNA was purified according to a standard phenol-chloroform procedure after enzymatic digestion. Five nanograms of linearized pSTK plasmid was cotransformed (by electroporation) at various molecular ratios (1:1, 1:5, 1:10) with linearized pShuttle plasmid into *Escherichia coli* BJ5183 (Stratagene/Agilent Technologies, Santa Clara, CA). Recombinant plasmids were screened by restriction enzyme digestion and subsequently transformed in *E. coli* XL-2 blue (Stratagene/Agilent Technologies) to avoid further recombinations/rearrangements.

Generation of HDAd vectors

To rescue and amplify HDAd vectors, 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) in accordance with the

FIG. 1. Schematic representation of novel plasmid family used to generate helper-dependent adenoviral (HDAd) vectors by homologous recombination. **(A)** Scheme of HDAd vector backbones used in this study. In all cases the vector backbone was released from the bacterial plasmid by means of *PmeI* sites located at both ends of the inverted terminal repeats (ITRs). The name of each plasmid and its cloning capacity are 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 *Escherichia coli* BJ5183 bacteria after transformation with linearized pShuttle plasmid and the pSTK plasmid. HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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 the 116cre cell line. Passage 0 (P0) is the transfection of 116cre cells with the HDAd backbone; P1, P2, P3, and P4 are subsequent viral amplifications (first to fourth, respectively). Representative fluorescence 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, $\times 100$. **(C)** Quantification of secretable alkaline phosphatase (seAP) in the culture medium of 116cre cells at each amplification step. **(D)** Quantification of seAP in the culture medium of HEK-293 cells transfected with CMV-seAP, elastase (Ela)-seAP, human insulin (hINS)-seAP, or null (irrelevant) plasmid. **(E)** Quantification of seAP in the culture medium of 266-6 murine acinar cells. **(F)** Quantification of seAP in the culture medium of transfected MIN6-B1 murine beta cells.

protocol described by Palmer and Ng (2003) (116cre cells were kindly provided by P. Ng, Baylor College of Medicine, Houston, TX). Briefly, a 6-cm culture dish of 116cre cells was transfected with the viral genome (passage 0, or P0), and 48 hr after HV infection the cells were harvested. Vectors were released by freeze-thawing and used to infect a second 6-cm plate of 116cre cells in the presence of HV (P1). P1 lysate was

used to infect a third 6-cm plate (P2), and the P2 lysate was used to infect a 15-cm plate (P3). Finally, the P3 lysate was distributed into 20 $\times$ 15 cm plates (P4), cells were harvested 48 hr later and lysed, and HDAd vectors were purified by passage through two cesium chloride gradients (one discontinuous and one continuous). Subsequently, desalting PD-10 chromatography columns (GE Healthcare Life Sciences, Little

Chalfont, UK) were used. Vectors were formulated in sterile phosphate-buffered saline (PBS) containing 10% glycerol and stored at -80°C until use. Titration of HDAd particles and determination of percentage of residual HV were performed by qPCR. Specific primers to amplify the human HPRT region were used to titer HDAd particles (HPRT forward primer, 5'-GGGCATAAAGGGTTTAAATGG-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 as follows: 3×10^{11} viral particles (VP)/ml for HDAd-CMV-GFP (1.5% HV), 4.8×10^{11} VP/ml for HDAd-ela-GFP (5.8% HV), and 2.7×10^{11} VP/ml for HDAd-hINS-seAP (1.6% HV).

Cell culture and transfection

The acinar cell line 266-6 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) (cat. no. CRL-2151) and was cultured with Dulbecco's modified Eagle's medium (DMEM; glucose, 4.5 g/liter) supplemented with 10% fetal bovine serum (FBS). Flasks and plates were treated with 0.1% pig skin gelatin for 15 min before use to avoid clumping. The cell line HEK-293 (Qbiogene/MP Biomedicals, Santa Ana, CA) was cultured with DMEM (glucose, 4.5 g/liter) 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 (glucose, 4.5 g/liter) supplemented with 15% FBS, 71 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$).

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 anesthetized 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 2×10^{10} VP diluted in PBS) was injected into the duct over approximately 1 min. At 1 min postinjection the microclamps and needle were removed. For systemic administration, 200 μl of 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) and guinea pig anti-insulin (Sigma-Aldrich, St. Louis, MO) antibodies. As secondary antibodies, biotinylated donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-guinea pig (Life Technologies, Carlsbad, CA) were used. Streptavidin-Alexa Fluor 488 (Life Technologies) and 3,3'-diaminobenzidine (DAB) were used

for fluorescence imaging and bright-field imaging, respectively. Sections were counterstained with Hoechst (Sigma-Aldrich) for nuclear counterstaining of fluorescent specimens. Hematoxylin and eosin staining was used for the bright-field images.

Quantification of transduction

Detection of GFP was performed in three pancreas sections per animal (three or four animals per group; sections 200 μm apart). Quantification of transduction of the exocrine pancreas was performed in 10 randomly selected 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 GmbH, Münster, Germany). 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⁺ cells of that islet.

Vector genome copy number quantification

Vector genome copy number was measured from frozen or paraffin-embedded tissues. DNA was isolated from paraffin blocks containing pancreas tissue, using a NucleoSpin FFPE DNA kit (Macherey-Nagel, Düren, Germany) according to kit specifications. Briefly, seven sections (thickness, 10 μm) were cut for each animal sample and the paraffin was dissolved. After paraffin dissolution, tissue was digested overnight with proteinase K in lysis buffer. DNA was then de-cross-linked, precipitated with ethanol, and purified in the kit columns. Total DNA from frozen tissues was isolated with a MasterPure DNA purification kit (Epicentre, Madison, WI) after overnight digestions in proteinase K (0.2 mg/ml).

Resulting DNA from paraffin-embedded or frozen tissues was diluted in distilled water to 100 ng of DNA per microliter. 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 Fig. 2): forward primer, 5'-CTGGTCTGCAGTTAGGAGATAC-3'; reverse primer, 5'-CAGAGGCTCAGAGAGGTTAAAT-3'; probe, 5'-ACTTCTTAGCCGTGTACCTTGGG-3'.

The final values were determined by comparison with a reference standard curve, built from serial dilutions of the linearized plasmids used for HDAd vector production spiked into 100 ng of nontransduced genomic DNA.

Measurement of serum alkaline phosphatase levels

Circulating seAP levels were determined from serum samples with placental seAP Tropix Phospha-Light chemiluminescence 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 pretreatment ensures inactivation of endogenous seAP but not of transgene-derived placental seAP, because 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 represent means $\pm$ SEM. Differences between groups were compared by unpaired Student *t* test. Statistical significance is indicated by a single asterisk (**p* < 0.05) and a double asterisk (***p* < 0.01).

Results

Novel plasmid family to generate HDAd by homologous recombination

The helper-dependent adenoviral genome should have a final size greater than 28 kb to allow efficient packaging in the adenoviral capsid (Parks and Graham, 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, 16, 19, and 20 kb) of human DNA from the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus were used as stuffer DNA in all plasmids, except for pSTK42 (Fig. 1A). Fragments of genomic DNA from the C346 cosmid were also used in pSTK129, pSTK120, and pSTK119 plasmids (Fig. 1A). All pSTK plasmids were constructed using pBluescript (Agilent Technologies) as the 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 pSTK plasmids is difficult because of the large size and 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 adenoviral (Chartier *et al.*, 1996) and 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 adenoviral ITR and the packaging signal were excised by *PmeI*–*EcoRV* digestion from pSTK129 and cloned into the *Apal* site of the p3T plasmid, this fragment being the left arm for homologous recombination. Next, pSTK129 was digested with *EcoRV* and *FspAI* and a fragment of 1.7 kb belonging to the HPRT stuffer DNA (depicted in gray in Fig. 1A and B) 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 B) 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 B, pShuttle EA1 was designed for homologous recombination with pSTK129 and pSTK134 and 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 carrying the transgene of interest was linearized

with a restriction enzyme cutting the bacterial backbone, and the pSTK plasmid was digested with *EcoRV* (site located between 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 an HDAd plasmid generated by standard ligation (pFK7) (Fig. 2). HDAd-CMV-RFP (named pEA4) expressed red fluorescent protein (RFP) under the control of the ubiquitous CMV promoter, HDAd-ela-GFP (pEA9) expressed GFP under the control of the pancreas-specific elastase promoter (Kruse *et al.*, 1993, 1995), and HDAd-hINS-seAP (pEA11) expressed the human placenta-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 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 pShuttle EA2 and homologous recombination was performed with pSTK119.

As shown in Fig. 2B, there was an increase in the number of GFP and RFP cells from P1 to P4, indicating correct amplification of HDAd vectors generated by homologous recombination. Surprisingly, GFP was expressed in 116cre cells under the control of the elastase (Ela) 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 cells as evidenced by an increase in seAP levels in the 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, Ela-seAP, and hINS-seAP constructs into HEK-293 cells, 266-6 (mouse acinar) cells, and MIN6-B1 (mouse beta) cells (Fig. 2D–F). Our data suggested that HEK-293 and 116cre cells are 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 transduction mediated by HDAd expressing GFP

The efficiency of FGAd vectors in transducing the pancreas via different routes has been investigated (McClane *et al.*, 1997a–c; Ayuso *et al.*, 2004; 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, and 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 and Muruve, 2003). In contrast, HDAds have demonstrated long-term persistence in several tissues in small and large animal models (Brunetti-Pierri and Ng, 2009). Here, we investigated the efficiency of transduction and the duration of transgene expression mediated by HDAd in the pancreas.

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

Next, a dose of 2×10^{10} VP of HDAd-ela-GFP vector was injected via the pancreatic duct into immunocompetent ICR mice. Animals were killed and analyzed as described previously. Widespread transduction of the pancreas 5 days postinjection was found, the acinar transduction being about 1.5% (Fig. 4A and B). Although GFP⁺ cells were significantly reduced both 15 and 30 days postinjection, positive cells were still maintained 30 days postinjection (Fig. 4A and B). 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 (Ornitz *et al.*, 1985; Jimenez *et al.*, 2011). Lymphocytic infiltration was detected mainly in the pancreas of mice 15 days postinjection, but some lymphocytes were also present 30 days postinjection, whereas minimal infiltration was detected 5 days postinjection (Fig. 4A).

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

Secretable alkaline phosphatase has been used as a marker transgene in several studies, mainly because it is

FIG. 3. Pancreatic transduction with HDAd-CMV-GFP administered via intraductal delivery. ICR mice were injected intraductally with 2×10^{10} viral particles (VP) of HDAd encoding the green fluorescent protein (GFP) reporter gene driven by the ubiquitous cytomegalovirus (CMV) promoter. Animals were analyzed 5, 15, and 30 days after injection. (A) Immunohistochemical analysis of GFP (green, top) and hematoxylin-eosin (H/E) staining of pancreatic sections (bottom). Arrows point to 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). Arrows point at double-positive cells. Results are expressed as means $\pm$ SEM; $n = 4-6$ animals per group. * $p < 0.05$ versus day 5. ND, not detected. Original magnification: (A) $\times 200$; (D) $\times 100$.

FIG. 4. Pancreatic transduction by HDAd-*ela*-GFP administered via intraductal delivery. ICR mice were injected intraductally with 2×10^{10} VP of HDAd encoding the GFP reporter gene driven by the pancreas-specific elastase promoter. Animals were analyzed 5, 15, and 30 days after injection. (A) Immunohistochemical analysis of GFP (green, *top*) and hematoxylin–eosin (H/E) staining of pancreatic sections (*bottom*). Arrows point to lymphocytic infiltration. (B) Quantification of GFP-positive exocrine cells per pancreas (%). (C) Immunohistochemical analysis of GFP (green) and insulin (red). Arrow points at a double-positive cell. Results are expressed as means $\pm$ SEM; $n = 4$ –6 animals per group. $**p < 0.01$ versus day 5. Original magnification: (A) $\times 200$; (C) $\times 400$.

secreted into the blood efficiently (Bartoli *et al.*, 2006; Riviere *et al.*, 2006; Shiraiwa *et al.*, 2007; Jimenez *et al.*, 2013). In addition, placenta-derived seAP is a thermo-resistant 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 placenta-derived seAP gene under a specific pancreatic promoter, that is, the human insulin promoter (hINS), were generated (Fig. 2). A dose of 2×10^{10} VP of HDAd-hINS-seAP vector 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 postinjection. On the other hand, seAP was not detected in the serum of mice treated with the same vector dose but delivered intravenously (Fig. 5A and B), demonstrating that (1) adenoviruses do not reach the beta cells via intravenous delivery, as previously observed (Ayuso *et al.*, 2004), and (2) the insulin promoter is not active in nonbeta 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) killed 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, which 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, that is, better transduction of the target organ with reduced systemic dissemination (Brunetti-Pierri *et al.*, 2009, 2012).

Here, we investigated the systemic dissemination of HDAd-hINS-seAP vectors administered via intraductal delivery in comparison with intravenous injection. As expected, on intravenous administration of HDAd vector 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 intraductally (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. S1; Supplementary Data are available online at www.liebertpub.com/hum).

The quantity of vector genomes found in the pancreas was low both in the intraductally and intravenously injected animals (Fig. 6A), although higher amounts of vector genomes were found in the animals injected intraductally (Fig. 6B). Despite the low vector copy number found in the pancreas, efficient transduction of this tissue was achieved

FIG. 5. Long-term expression of seAP in immunocompetent mice after HDAd-hINS-seAP intraductal delivery. C57BL/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 pancreas-specific human insulin promoter. seAP levels were monitored periodically in serum samples from these mice as indicated in Materials and Methods. (C and D) Hematoxylin–eosin staining of pancreatic (top) and liver (bottom) sections of C57BL/6 mice (C) and ICR mice (D) 8 months after HDAd-hINS-seAP delivery. ID, intraductal; IV, intravenous; Con, healthy control mice ($n=9$ or 10 per group). Original magnification, $\times 100$. * $p < 0.05$ versus IV; ** $p < 0.01$ versus IV.

in the intraductal group but not in the intravenous group (Fig. 6C; and Fig. 5A and B).

We also investigated the persistence of HDAd vector genomes at several time points postinfection in the pancreas of animals injected intraductally 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 extent in HDAd-ela-GFP mice (Supplementary Fig. S2A and B). Persistent vector genomes were detected in the pancreas 8 months after intraductal administration of HDAd-hINS-seAP vector (Supplementary Fig. S2C).

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

Discussion

Promising results have been obtained for gene therapy of hemophilia B (Brunetti-Pierri *et al.*, 2005a), hyperbilirubinemia (Dimmock *et al.*, 2011), glycogen storage disease type Ia (Crane *et al.*, 2012), and glioblastoma (Muhammad

FIG. 6. Vector genome bio-distribution in ICR mice after intraductal or intravenous administration of HDAd. A dose of 2×10^{10} VP of HDAd-hINS-seAP was administered by intraductal (ID) injection or intravenously (IV) to ICR mice. Two weeks postinjection the vector genomes were quantified by qPCR in several tissues (A and B) and seAP levels were measured in the serum (C). Values shown represent means $\pm$ SEM ($n=4$ or 5 animals per group). * $p<0.05$ versus ID; ** $p<0.01$ versus ID.

et al., 2013) using helper-dependent adenoviral vectors targeting the liver and the brain. In the current work we investigated the usefulness 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 is blocked in mice (Ayuso *et al.*, 2004) or when the vectors were delivered into clamped pancreatic vessels of dogs (Ayuso *et al.*, 2006). It is noteworthy that adenoviruses can also be retrogradely injected into the common bile duct to transduce the pancreas. Using this locoregional route, efficient and selective transduction of the pancreas was achieved with FGAd and also oncolytic adenoviral vectors (José *et al.*, 2013). Antitumoral responses were stronger by ductal viral application than intravenously, in line with the 38-fold increase in pancreas transduction observed on ductal administration (José *et al.*, 2013). Similarly, AAV vectors transduced the pancreas highly efficiently and persistently after delivery via the pancreatic duct (Wang *et al.*, 2006; Jimenez *et al.*, 2011). Taken together, 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, this efficiency being significantly lower compared with that of AAV vectors (Jimenez *et al.*, 2011). One way to increase 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 by novel technologies (Palmer and Ng, 2008). However, a potential problem with the use of 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 due mostly to liver and spleen transduction, and in particular is due to scavenging by macrophages and their activation. Importantly, here we have shown a significant reduction in systemic HDAd vector dissemination on intraductal delivery with minimal detection of vector genomes in the liver, lungs, and spleen compared with the intravenous route. Consistently, no expression of GFP was detected in the liver on 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, because the latter route resulted in strong expression of the marker gene in hepatocytes (Supplementary Fig. S3) unless a pancreas-specific promoter was used (Supplementary Fig. S4).

One of the most desired properties of HDAd vectors is the ability to mediate long-term transgene expression in immunocompetent animals. However, 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 postinjection, by which time transgene expression had been lost. At this time point and also 60 and 90 days postinjection, 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). Taken together, 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 -galactosidase (β -Gal) in 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 vectors are able to elicit significant transgene-directed T cell and antibody responses (Weaver *et al.*, 2009); whereas Kron and colleagues 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 the T cell-mediated immune response, can inhibit the CMV promoter (Harms and Splitter, 1995). Although silencing of the CMV promoter cannot be completely ruled out, our data showing a reduction in the number of vector genomes in the pancreas 30 days postinfection in animals injected with HDAd-CMV-GFP suggested that immune-mediated destruction of transduced cells was the most likely explanation for transgene loss. In HD-*ela*-GFP-injected animals, vector genome reduction in the pancreas 30 days postinfection was less pronounced, which is in agreement with later lymphocytic infiltration of the pancreas of these animals compared with HDAd-CMV-GFP-injected animals.

Importantly, in using a mammalian gene such as human seAP we did not observe loss of transgene expression in parallel with detection of HDAd vector genomes in the pancreas of animals 8 months postadministration, 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 for the lack of immunogenicity. Moreover, the fact that seAP expression was limited to beta 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 adenoviral vectors (Pastore *et al.*, 1999).

We would also like to highlight the sensitivity of seAP as a reporter transgene. Only 1% of beta cells transduced with HDAd vectors was sufficient to produce detectable seAP levels in serum. seAP is also convenient because it can be measured longitudinally (i.e., at various 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 (pShuttle EA1 and pShuttle EA2) mediated homologous recombination with the HDAd backbone (pSTK) at the expected sites. Moreover, HDAd vectors generated by this system propagated normally in 116cre 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 with low systemic vector dissemination. These data suggest that pancreatic gene transfer/therapy approaches for the treatment of diabetes mellitus, chronic pancreatitis, and 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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