A novel gene delivery method transduces porcine pancreatic duct epithelial cells

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INTRODUCTION
Cystic fibrosis (CF) is a multisystem disease caused by mutations in the gene encoding CF transmembrane conductance regulator (CFTR). CFTR is expressed in many epithelial cells, including pancreatic ducts, and functions as an apical membrane anion channel. Genetic mutations in CFTR determine the exocrine pancreatic function in CF. In patients with CF who carry two severe mutations that severely affect CFTR function, the pancreatic damage starts in utero. In these individuals, the damage continues after birth and they become pancreatic-insufficient at young ages. Patients with sufficient pancreatic function carry a mild mutation on at least one allele and have residual CFTR activity (~10% of all CF patients). Patients with pancreatic sufficiency are prone to recurrent pancreatitis attacks and progressive decline in the exocrine pancreatic function as a consequence.

Despite treatment with pancreatic enzymes to prevent severe malnutrition, exocrine pancreatic insufficiency in CF tracks with delayed growth, accelerated progression of lung disease, and CF-related diabetes, all associated with increased morbidity and mortality. Preserving the exocrine pancreatic function in CF may improve disease outcomes. Currently, there are no treatments to prevent the pancreatic disease progression in CF.

Designing therapies for CF pancreatic disease has been challenging because the pancreas is not easily accessible in humans, and mice models do not develop pancreatic disease typical of CF. Newborn CF pigs have pancreatic disease similar to patients with CF and the disease progresses over time, as it does in humans. Therefore, the CF pig model creates an opportunity to study gene therapy for pancreatic disease. To date, there are no studies assessing the transduction of pig pancreas.

The available techniques to transduce cells in the pancreas of mice and rats (direct pancreatic injection, retrograde pancreaticobiliary duct delivery or systemic delivery with temporary clamping of portal vein, hepatic artery, bile duct) are invasive, induce severe pancreatic inflammation and toxicity and are not desirable for human studies. Other methods are ineffective: intravenous delivery of adenovirus vectors does not transduce pancreatic cells because the liver rapidly removes the virus from circulation. In general, adenoviral vector-directed gene transfer to the pancreas has been limited by inflammation and transient expression. Adeno-associated virus (AAV) vectors are attractive because of their low immunogenicity, excellent safety record and long-term transgene expression in non-dividing cells, even in the absence of genome integration. Still, the experience with delivering AAV vectors to the pancreas is limited. Moreover, the delivery methods are usually invasive and mainly transduce the acinar cells and islets of mice, not the pancreatic duct epithelial cells in which CFTR is expressed.

The goal of this work was to express genes in the pancreatic duct epithelial cells of wild-type (WT) pigs and to create a framework for future studies for CFTR gene delivery to the pancreatic ducts of CF pigs. AAV9 vector delivery via the celiac artery, the vessel that supplies major branches to the pancreas, efficiently and stably transduced pancreatic duct epithelial cells. This is the first study showing the expression of transgenes in pig pancreatic duct cells.
RESULTS

IV injection of the AAV9 vector does not transduce the pancreas in pigs

Our goal was to target the pancreas and primarily CFTR-expressing pancreatic duct epithelial cells, using an efficient and minimally invasive technique. We chose pigs because CF pigs lacking the CFTR function exhibit defective anion transport and replicate the multisystem disease observed in humans with CF, including pancreatic disease.22–27

We delivered AAV9CMV.sceGFP (green fluorescent protein) (2.4 × 10^{12} viral genome particles (vg) per animal, n = 2) intravenously (ear vein) to 1-day-old pigs and we observed no gene transfer to the pancreas, 1 month after the injection (data not shown). Therefore, systemic venous delivery did not transduce the pancreas in newborn pigs.

Injection of the celiac artery as a novel method to deliver transgenes to the pancreas of newborn pigs

We next assessed vector delivery via the celiac artery, the vessel that supplies major branches to the pancreas in humans and pigs.22 Shortly after birth (24–48 h), the celiac artery can be easily accessed via the umbilical arteries (Figure 1). The AAV9 vector was administered to the celiac artery of newborn pigs and all pigs tolerated the procedure well without complications. After the procedure, piglets recovered uneventfully and received standard care.

AAV9 gene delivery via the celiac artery did not induce pancreatic inflammation in pigs

Adenoviral vector-directed gene transfer to the pancreas is limited by inflammation and transient expression of the genes in rodents.28–31 However, AAV vectors typically have low immunogenicity. To determine whether AAV9 caused an immunogenic response in pigs, we monitored their activity level, food intake and weight gain on a daily basis. We observed no differences between vector and vehicle-treated pigs. One and 3 months after vector delivery, animals were euthanized and pancreata were isolated. We examined the pancreatic histology of pigs that received AAV9 at birth and compared this to the control pigs. The pancreas had normal architecture with no infiltrating inflammatory cells after vector delivery (Figure 2).

GFP is expressed in porcine pancreatic duct epithelial cells following AAV9 vector delivery to the celiac artery

Although AAV vectors have been used to target other organ systems, there is limited information on their delivery to the pancreas. In general, gene transfer to the pancreas has been carried out in vitro and/or on islet cells of rodents.43–46 There are no data reported using AAV vectors in pigs. To determine whether the GFP reporter gene was expressed in pancreatic duct cells following the delivery of AAV9 vector to the celiac artery of newborn pigs, we used immunofluorescence (IF), immunohistochemistry (IHC) and (reverse transcription polymerase chain reaction) RT-PCR. Figures 3a—f summarizes our findings in pigs euthanized 1 month after receiving 2.4 × 10^{12} vg of AAV9CMV.sceGFP (n = 7) or vehicle per animal. Supplementary Figure S1 shows IF images from pigs that received various doses of the AAV9 vector or vehicle and followed for 1–3 months.

One month after delivering the AAV9 vector to the celiac artery, we found GFP expression in pig pancreatic ducts, including the intercalated and intralobular ducts (Figures 3a, c and e; Supplementary Figure S1) that normally have high levels of CFTR.47–49 There was no staining detected if the primary antibody was omitted (IHC) (Figures 3d and f), confirming that the antibody staining was specific to GFP. Vehicle-treated animals were not immunoreactive for GFP (Figure 3b). Gene expression was dose-dependent and persisted 3 months after treatment (last time point tested) (Figure 4). GFP expression shown using IF and IHC in pig pancreas was confirmed with PCR both at 1- and 3-month time points (Figure 5a). We detected transduction of ~10% of the cells of the pancreas, predominantly ductal epithelial cells, 2 months after delivery of the AAV9CMV.eGFP vector (non-self complimentary form), using 2.4 × 10^{12} vg (n = 3). Thus, the delivery of the AAV9 vector to the celiac artery in newborn pigs effectively transduces the pancreatic duct epithelial cells.

AAV9 vector delivered to the celiac artery of newborn pigs transduces CFTR-expressing pancreatic duct epithelial cells

Studies in human and pig samples have shown that CFTR is expressed at high levels in the pancreas and localizes to the pancreatic duct epithelia.27,47–49 To determine whether GFP was expressed in CFTR-expressing pancreatic duct epithelial cells following AAV9 delivery, we immunolocalized CFTR in transduced tissues (Figure 6a). CFTR was expressed on the apical side of duct epithelia, and CFTR and GFP colocalized within the same cells. These results confirm that our technique transduces CFTR-expressing duct cells in the pancreas.

AAV9 vector transduces pancreatic polypeptide-secreting cells of the islets

AAV9 transduces β cells and, to a lesser degree, α cells in mice;32 however, it is not known whether porcine pancreatic cells are susceptible to AAV9 transduction. To examine the pancreatic cell subtypes transduced with our technique, we immunostained pancreas sections with antibodies against amylase (acinar cell

Figure 1. Celiac artery catheterization via umbilical arteries. (a) In newborns, celiac artery can be reached by placing a catheter (arrow, green) into the umbilical arteries, which connect to the aorta. The catheter is then advanced to the celiac artery. (1) aorta; (2) right iliac artery; (3) umbilical cord (one vein and two arteries); (4) pancreas; (5) hepatic artery; (6) splenic artery; (7) celiac artery with catheter; (8) umbilical artery; (9) left femoral artery. (b) Angiography confirming cannulation of the celiac artery (arrows). Vector or vehicle was injected and the catheter was flushed with normal saline.
Figure 2. AAV9 gene delivery does not induce pancreatic inflammation in pigs. Thirty (a, b) and ninety days (c, d) after the celiac artery injection of AAV9CMV.sceGFP (2.4 × 10^{12} vg per animal), pancreas sections were obtained. The pancreas had a lobular architecture, with ducts (arrow), acini (*) and islet cells (block arrow). There were no inflammatory cells (H&E stain). a, c × 10 magnification, bar = 50 μm; (b), (d) × 60 magnification, bar = 20 μm.

Figure 3. AAV9 transduces porcine ductal epithelial cells. Pancreas sections 30 days after newborn pigs received AAV9CMV.sceGFP (a, c–f) (2.4 × 10^{12} vg per animal) or vehicle (b) into the celiac artery. Immunofluorescence (a, b) and immunohistochemistry (c–f) images are shown. Arrows point to intralobular (larger) ducts, arrowheads point to intercalated (smaller) ducts. Panels c and d as well as e and f are serial sections from the same animal, primary antibody is omitted in d and f. Magnification: a and b, × 20; c and d, × 10 (scale bar = 100 μm); e and f, × 60 (scale bar = 20 μm). Green: GFP, Blue: DAPI nuclei.
Delivering the AAV9 vector to the celiac artery transduces other organs

As our technique involves a systemic injection of a vector with a CMV (cytomegalovirus) promoter, other organs could also be transduced. The celiac artery supplies blood to the stomach, duodenum, spleen, liver and gallbladder, and the vector may also enter the systemic circulation and reach other organs. To determine whether other organs were also transduced following the celiac artery injection of the AAV9CMV.sceGFP vector, we performed end point RT-PCR for GFP 30 days after the injection. The organs that are transduced by our technique are shown in Figure 5b. The liver, gallbladder, cystic duct and spleen receive blood supply from the celiac artery and were transduced by our technique. Interestingly, the organs that receive the blood supply from the celiac artery, such as the stomach and the duodenum, were not transduced. The transduction of other organs (salivary gland, trachea, lung, vas deferens and ileum) typically involved in CF may be advantageous for treating this systemic disease.

**DISCUSSION**

In this study, we describe a novel, safe and minimally invasive gene delivery technique to efficiently express a reporter gene in the pancreatic duct epithelial cells of pigs, an animal species that has a CF model available. This is the first study showing efficient transduction of pig pancreas with a gene-transfer vector.

The pancreas is a retroperitoneal organ and is difficult to access. The techniques that deliver genes to the pancreas of mice and rats involve injecting the pancreatic parenchyma or the pancreatic duct or giving it systemically in conjunction with laparotomy and clamping the portal vein, the hepatic artery or the bile duct. Therefore, these methods are invasive and are not easily translated to humans. A major advantage of our technique is the ease with which it is performed. As the umbilical artery is patent in newborn pigs for 24–48 h after birth, it allows easy, noninvasive (no surgical cutdown needed) access to the aorta, celiac artery and the pancreatic arterial supply. Umbilical artery catheterization is commonly performed in humans and is well-tolerated by even premature, very low birth weight neonates. Once the umbilical vessels are no longer accessible, the celiac artery can be catheterized via the femoral artery. Therefore, our method has the potential to be translated to humans.

In general, viral vectors delivered to the venous system do not efficiently transduce the pancreas. This is probably because the vector is removed from the circulation before it reaches the pancreas. Indeed, we have not observed pancreatic gene expression following the IV delivery of the vector. Our technique circumvents this problem by directly delivering the vector to the arterial blood supply of the pancreas, using a minimally invasive approach. The technique is well tolerated by the animals and leads to efficient transgene expression, 1 and 3 months after delivery.

Inflammation and transient transgene expression have been the major problems with delivering adenoviral vectors to the pancreas. Inflammation has not been observed with AAV vectors, although the experience with delivering AAV vectors to the pancreas is limited. We observed no pancreatic inflammation in our model 1 and 3 months after gene delivery, confirming that the AAV vectors are suitable for use in pancreatic gene-transfer studies.

There is limited information on delivering AAV vectors to the pancreas. In general, gene-transfer studies to the pancreas have been carried out in vitro and/or on islet cells of rodents. Serotypes 1, 2, 5, 6 and 8 have been used in vitro and in vivo in mouse pancreases. Transduction of ductal cells has been reported in mouse pancreas with AAV6 and AAV8, however,
the vectors were delivered via the pancreatic duct or direct pancreatic injection. The colocalization of the transgene with CFTR was also not examined. Our studies confirm that AAV serotype 9 is an efficient vector to transduce the pancreas. We have not explored the other serotypes.

Previous studies with AAV delivery to the mouse pancreas reported the AAV transduction of acinar cells and islets (mainly β cells), not the pancreatic duct cells where CFTR is expressed. Delivering transgenes to CFTR-expressing pancreatic ducts is a novel and exciting finding of this study. This method has the potential to transfer the CFTR gene to the pancreas of humans with CF. In addition, this approach might be used to target genes that control cell proliferation and survival in humans with pancreatic ductal adenocarcinoma or have applications for other genetic or acquired diseases of the pancreas.

Another interesting finding of this study is the expression of transgenes in pancreatic polypeptide-expressing cells. Whereas the exact physiological role of PP is not determined, the plasma levels of this hormone are reduced in humans with CF and in patients who develop diabetes secondary to chronic pancreatitis. The lack of a PP response to hypoglycemia or secretin confirms the exocrine pancreatic dysfunction in humans with CF. It is not known whether PP has a role in CF-related diabetes. A future goal is to transduce the CF pig pancreas with a shortened CFTR complementary DNA packaged in the AAV9 capsid and examine whether gene therapy will prevent the progression of pancreatic destruction. It will be interesting to learn whether CFTR gene transfer to the CF pig pancreas will have an effect on PP levels and insulin secretion.

One limitation of our study is the broad expression of transgenes in other organs. The vector likely reaches other organs by entering the systemic venous circulation. Interestingly, whereas the stomach and duodenum receive their blood supply from the celiac artery, they were not transduced, suggesting that other factors such as AAV9 receptor-mediated uptake may also be involved. Our technique may be advantageous for a systemic disease such as CF that involves multiple organs. For future studies, a promoter that is more specific to the pancreas (that is, pdx1) may achieve more targeted expression.

In summary, we report a novel, efficient and well-tolerated gene-delivery technique to the pancreatic duct epithelial cells of a large animal species that has a CF model available. Future studies will explore the utility of this technique to restore CFTR function, anion transport and prevent pancreatic disease progression in CF pigs. Successful execution of gene therapy in CF pigs would provide an important step towards translational studies in humans with CF.

MATERIALS AND METHODS

Virus preparation

AAV9CMV.sceGFP (self-complementary genome) or AAV9CMV.eGFP was produced by triple-plasmid co-transfection of human HEK 293 cells and...
purified by Mustang Q membrane cassettes after iodixanol gradient centrifugation. The vectors were dialyzed using 7000 MWCO Slide-A-Lyzer Mini Dialysis Units (Pierce Cat no. 69560 (10–100 µl) Rockford, IL, USA), in a 100:1 buffer (HyClone Cat no. RR10417.01) to sample ratio. The dialysis unit was then placed in a flotation device and dialyzed at 4 °C for 60 min using a low-speed setting on a stir plate. The sample was collected and kept on ice until delivery.

Animal procedures
All studies were approved by the University of Iowa Animal Care and Use Committee. Newborn pigs (Sus scrofa) were obtained during the first 24 h of life, when the umbilical cord was still present. The procedure was performed by an interventional pediatric cardiologist (AD). He had previously developed a minimally invasive and innovative method for transcatheter intervention of the ductus arteriosus by cannulating the umbilical artery in newborn pigs.²⁵ We modified this technique by selectively canulating the celiac artery, which is the vessel that supplies major branches to the pancreas in humans and pigs.²⁶ Shortly after birth (24–48 h), the celiac artery can be easily accessed via the umbilical arteries that extend into the umbilical cord (Figure 1a).

Piglets were anesthetized using spontaneous mask ventilation with isoflurane. Pulse oximetry, breath CO₂, heart rate and body temperature were monitored throughout the procedure. IV hydration was maintained with 10% dextrose infusion through a peripheral vein. The animal was placed in the right lateral decubitus position. The entire procedure was performed under sterile technique. A previously flushed 3.5 Fr. single lumen arterial catheter (Kendall, Argyle, Tyco Healthcare Group, Mansfield, MA, USA) was advanced into the umbilical artery to 20 cm, free flow of arterial blood was obtained and the catheter was flushed with saline. Position in the thoracic aorta was confirmed using fluoroscopy. Under fluoroscopic control, the catheter was exchanged over a 0.021 pre-wetted guide wire (Argon Medical Devices, Inc Athens, TX, USA) for a flushed 4 Fr. introducer (Cordis, Johnson & Johnson, Miami, FL, USA). The dilator was removed and a 4 Fr. Cobra 1 (C1) Glidecath (Terumo Medical Corporation, Somerset, NJ, USA) was advanced over the wire and placed in the descending aorta. The catheter was flushed with saline after removing the wire. The catheter was slowly withdrawn below the diaphragm and the celiac artery was cannulated. Angiography confirmed the cannulation (Figure 1b). AV9CMV.scGFP (2.4 × 10¹² vg per animal; 1.2 × 10¹² vg per animal; 2.4 × 10¹¹ vg per animal; 6.1 × 10¹¹ vg per animal; n = 1 for all time points and doses, except n = 2 for 6.1 × 10¹² vg at 1 month, n = 7 for 2.4 × 10¹¹ vg at 1 month) or AV9CMV.mGFP (2.4 × 10¹² vg per animal; n = 3 at 2 months) were injected into the celiac artery and the catheter was flushed again with 5 ml normal saline. The vehicle was given to two animals as control and they were killed at 1 and 3 months.

After the procedure, piglets recovered uneventfully and received standard care. During the first 24 h, the piglets were fed a liquid colostrum supplement (Manna Pro, Saint Louis, MO, USA) via syringe every 2 h followed by milk replacer (Multi-species Milk Replacer, Carpentersville, IL, USA) via syringe every 4 h until being competent to feed independently. The piglets were transitioned to pelleted feed at ~2 weeks of age. One and 3 months after vector delivery, animals were euthanized using intracardiac Euthasol injection (90 mg kg⁻¹), followed by bilateral thoraco-tomy. The animals were not kept beyond 3 months of age because they become very large (>100 lbs) and challenging to handle in the animal care facility.

Necropsy and tissue harvesting
One or three months after injection, the animals were sedated with intramuscular injection of Ketamine (20 mg kg⁻¹) and Xylazine (0.2–2.2 mg kg⁻¹) and euthanized as described above. A full necropsy was performed and tissues were collected. Tissues were placed in 4% paraformaldehyde and fixed for 24–48 h. Following fixation, tissues were either processed and paraffin-embedded or placed through a series of sucrose gradients (10, 20 and 30%) for cryoprotection and snap-frozen.

IHC staining
Frozen tissue sections were cut (10 µm) and fixed in 10% ice-cold zinc formalin for 5' and washed with dH₂O. Sections were then immersed in Phosphate-buffered solution (PBS) for 5' and transferred into 0.2% Triton-X for 10 min for permeabilization. Sections were washed in PBS × 3 for 5 min each. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide (H₂O₂) at for 8' and washed in PBS × 3 for 5' each. Sections were blocked in 5% normal goat serum for 30' at room temperature (RT) and incubated at RT with primary (rabbit polyclonal anti-GFP, 1:400) for 1 h, followed by secondary antibody (Envision plus Rabbit) for 30'. Signal development was performed using a chromogen diaminobenzidine solution for 10 min and washed in running tap water for 10'. Tissues were counterstained in Harris Hematoxylin for 20', transferred back under the running tap water for 5 min, dehydrated through graded alcohols, cleared in xylenes and mounted.

IF staining
Frozen tissue sections were cut (10 µm) and fixed in 10% cold Z-fix for 5'. Sections were washed in tap water and then placed in three washes of PBS for 5' each. Tissues were permeablized in 0.2% Triton-X-100 for 10' and washed in PBS × 3. Nonspecific background staining was blocked using a 5% normal goat serum for 30'. Sections were incubated with primary antibody 1:400 anti-GFP (Abcam GRB 722-1, Cambridge, MA, USA) at 4 °C overnight, followed with secondary antibody (Alexa-flour 488) for 30' at RT. Slides were washed with PBS × 3 and were mounted with Vectashield and DAPI. Ten random pancreatic fields (20 × magnification) were assessed per animal and %GFP-positive cells were calculated by counting GFP expressing divided by the total number of cells in the field.

End point RT-PCR
End point RT-PCR was performed as a confirmation of GFP presence from tissues collected during necropsy, snap-frozen in liquid nitrogen and stored at −80 °C. The tissues were homogenized (no. 03-392-106 grinder, 0.5 ml mincing size; Fisher Scientific, Pittsburgh, PA, USA) and RNA was extracted using Qiagen RNeasy Lipid Tissue Kit (no.74084; Qiagen, Valencia, CA, USA) with the optional DNase digestion step performed to prevent genomic DNA contamination. Following RNA extraction, all samples were measured for RNA concentration using NanoDrop 1000 (Thermo Scientific, Rockford, IL, USA). Samples were randomly selected to obtain RNA integrity numbers using Agilent 2100 bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). RNA integrity numbers ranged from 7.4 to 9.2, indicating minimally degraded RNA suitable for downstream applications. Reverse-Transcriptase RT-PCR was performed using SuperScript VILO Master Mix (Cat. no. 11750500, Invitrogen, Grand Island, NY, USA), 1000 ng starting RNA concentration, and UltraPure RNase/DNase-Free distilled water (Cat. no. 10977015, Invitrogen). The thermal cycler (Product No. PTC-1148C, Bio-Rad, Hercules, CA, USA) settings were 25 °C for 10 min, 42 °C for 1 h and 85 °C for 5 min. End point RT-PCR was then performed on the complementary DNA synthesized using HotStartTaq Master Mix Kit (Cat. no. 203446, Qiagen), 10 µM eGFP forward primer 5'-ACGTAAACGCGCACAAAGTC-3', 10 µM eGFP reverse primer 5'-AAGTATGCTGTCGTTGATG' 3' (Integrated DNA Technologies, Coralville, IA, USA). A 1.5% agarose gel was prepared and the samples run at 120 V for 30 min.

Statistics
To measure transduction efficiency, 10 random pancreatic fields (20 × magnification) were assessed for all time points and concentrations (IF) (n = 1 for all time points and doses, except n = 2 for 6.1 × 10¹² vg at 1 month and n = 7 for 2.4 × 10¹¹ vg at 1 month). % GFP-positive cells were calculated by counting GFP-expressing cells divided by the total number of cells in the field. Data were presented as the average of individual data points.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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MA Griffin et al

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