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Portal Vein Delivery of Viral Vectors for Gene Therapy for Hemophilia

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Abstract

The liver is a very complex organ with a large variety of functions, making it an attractive organ for gene replacement therapy. Many genetic disorders can be corrected by delivering gene products directly into the liver using viral vectors. In this chapter, we will describe gene delivery via portal vein administration in mice and dogs to correct the blood coagulation disorder hemophilia B. Although there are multiple delivery routes for both viral and non-viral vectors in animals, portal vein administration delivers vectors directly and efficiently into the liver. Complete correction of murine hemophilia B and multi-year near-correction of canine hemophilia B have been achieved following portal vein delivery of adeno-associated viral (AAV) vectors expressing factor IX from hepatocyte-specific promoters. Peripheral vein injection can lead to increased vector dissemination to off-target organ such as the lung and spleen. Below, we will describe portal vein injection delivery route via laparotomy.

Keywords

Liver; Gene therapy; Portal vein; Viral vectors; AAV

1 Introduction

The goal of an in vivo gene transfer protocol for correction of human disease is optimal delivery and expression of the therapeutic gene in the target organ while minimizing dissemination to other organs, thereby increasing efficacy and minimizing possible immune reactions or other toxicities. Gene transfer to hepatocytes is very effective not only for correction of liver disease but also for systemic delivery of therapeutic proteins. For example, hepatocytes are the normal site of synthesis for several coagulation factors; and therefore, hepatic gene transfer has resulted in high therapeutic levels of factor VIII (FVIII) and factor IX (FIX) in several animal models, including mice, dogs, and nonhuman primates [1 – 6]. Stable therapeutic expression for many years, in some cases for more than a decade, in hemophilic dogs has been observed (unpublished data and refs. 2, 7–9). Importantly, hepatocytes are capable of producing and secreting high levels of biologically active

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[4]Presence of inhibitory antibodies can be detected by Bethesda assay, where hemophilic mouse plasma is mixed with normal mouse plasma 1:1 ratio. This mixture is incubated for 2 h at 37 °C. After addition of FIX-deficient human plasma and CaCl, time of clot formation is measured with fibrometer. Bethesda inhibitor titer is equivalent of the dilution of mouse plasma sample, calculated from a standard curve obtained from twofold serial dilution of normal human plasma.

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coagulation factors into the blood. Furthermore, optimal liver gene transfer using adeno-associated viral (AAV), lentiviral, and also other vectors can induce immune tolerance to the transgene product, in part via a regulatory T cell response [10 – 13] (*see* Note 1). Finally, liver gene transfer for correction of the hemophilia B has been successfully translated to human treatment in recent clinical trials [14]. In this chapter, we will describe gene transfer for correction of murine and canine hemophilia B using recombinant AAV vectors via portal vein delivery (*see* Note 2).

AAV vectors have a single-stranded or self-complementary (sc) DNA genome and are devoid of viral coding sequences [15]. Use of a strong hepatocyte-specific enhancer/promoter combination is recommended for liver gene transfer [14, 16]. There are multiple serotypes of AAV with distinct tissue tropism. For example, AAV8 can be effectively delivered to hepatocytes by peripheral vein injection such as the tail vein of a mouse [14, 17, 18]. Other serotypes, including AAV2 and AAV5, require infusion more directly into the blood supply of the liver, which can be accomplished by administration into the portal vein, mesenteric vein, or hepatic artery [5, 6, 16, 19, 20]. In mice, injections into the splenic capsule may be similarly suitable (but only at vector doses of 10^{11} vector genomes [vg] or higher, as the spleen may sequester too much of the vector at lower doses). Capsid variants such as AAV2 devoid of surface-exposed tyrosine residues further improve gene transfer [21]. Use of cDNAs optimized for mammalian codon usage can further improve therapeutic expression [14, 18, 22, 23]. Generally, resulting expression levels are vector dose dependent. For AAV2, a single portal vein infusion of 10^{12} vg/kg to FIX-deficient mice or dogs resulted in long-term correction of hemophilia B, without eliciting any types of toxicity [5–7].

Prior to approval of a gene therapy protocol for clinical studies in humans suffering from genetic disorders, the approach has to be thoroughly investigated in a suitable animal model. Mice have been extensively used as a model for hepatic gene transfer via portal vein injection to treat numerous genetic disorders, such as hemophilia A and B, α1 antitrypsin deficiency, and OTC (ornithine transcarbamylase) deficiency [13]. Mice have the advantage of being easily accessible, having a high reproduction rate, and offering the ability to perform batch surgeries without a need for a separate dedicated facility. Recombinant DNA technology allowed the hemophilia research community to generate a large number of different knockout mice and transgenic mice, lacking FVIII or FIX or expressing nonfunctional forms of the protein [24, 25]. Therefore, one can test the risk of an immune response to the therapeutic gene product as a function of the underlying mutation, using gene mutations described in humans with hemophilia. However, there are also certain disadvantages such as limited ability to study the long-term effects of the gene transfer and lack of scale up to an animal of a size more similar to that of humans. Using an alternative large animal model, a single injection of AAV-FIX into the portal circulation, on canine

[1]C57BL/6 mice typically show the highest level of transduction with AAV vectors in the liver, resulting in high systemic expression of FIX, while BALB/c mice show slightly lower transduction efficiency. Other strains, such as C3H mice, may show substantially reduced transduction efficiency and thus require higher vector doses to achieve similar levels of transgene expression.

[2]Portal vein has access to two thirds of the liver, which is a normal site of expression of FIX. Following hepatic gene transfer, FIX can be detected in the circulation by ELISA from 1 week following gene transfer and persist for >1 year or even the life span of the mouse. Antibodies specific to human and canine FIX (that do not cross-react with murine FIX) are used for antigen capture and are commercially available.

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hemophilia B models (Chapel Hill dogs), resulted in sustained expression of canine FIX for over 10 years at levels between 1 and 10 % [5, 6, 8, 26].

When choosing between mouse and canine models of hemophilia B for hepatic-based gene therapy studies, several issues need to be considered. The Chapel Hill strain of hemophilia B dogs has a missense mutation that leads to the absence of detectible FIX activity or antigen [27, 28]. Recombinant AAV-mediated gene therapy in these hemophilia B dogs has been successful for multiple years without adverse events, albeit at low but steadily improving levels of expression [5, 6, 26]. Thus the success of gene therapy can be better assessed in dogs since transgene expression seems to be lasting longer than the life span of mice. Hemophilia B dogs have on average 5 spontaneous bleeds/year in soft tissues or joints, a bleeder phenotype mirrors that found in human hemophilia B [7, 29]. This severe bleeding diathesis also provides a metric for detecting a change in phenotype following gene therapy. In general, hemophilia B mice do not bleed spontaneously, so assessing change in bleeder phenotype is more difficult. To date, inhibitor formation in this outbred hemophilia B dog strain has not been noted with portal vein approach. In some of the mouse strains, inhibitor formation to the coagulation proteins has been noted but newer mouse models may help understand this issue better [25] (*see* Notes 3–[5]). Also, dogs tolerate multiple blood draws better than mice (*see* Note 6). Frequent blood sampling is essential in these studies since the goal to express coagulation proteins in the systemic circulation over time. It is also helpful to have large volumes of plasma (~1,000 ml or more) drawn over time to determine the extent of posttranslational modification of transgene-expressed FIX relative to wild-type FIX. In contrast to rodents, the results of experiments in the hemophilia B dogs have been more predictive of outcomes in humans than those performed in other species. We have shown that FIX can be given subcutaneously or via the airways in our hemophilia B dogs, administration routes that could reduce or obviate the need for needles and venipuncture in hemophilia B patients and justify extrahepatic expression in patients with severe liver disease in whom a portal vein approach may be contraindicated [30]. As a direct result, human trials of subcutaneous and inhalational administration of recombinant FIX are being considered. FIX is not bioavailable by these routes in mice. Also, FIX infused into hemophilia B dogs had a comparable half-life to that found in humans [31 – 33], whereas mice had a markedly shorter half-life [19]. Had either the extravascular administration or half-life studies of FIX only been performed in mice, human trials may not have been pursued. Collecting semen from dogs, a procedure that is extremely difficult in mice, is used to assess the risk of germline transmission of gene therapy vectors [34]. These differences are the basis for many investigators, and advisory boards regard these dogs as an essential national resource for preclinical testing and long-term follow up of new treatments for the

[3]Portal vein infusion of AAV-FIX in mice can give weak antibody response to the viral particles, which can appear in the peripheral blood days after gene transfer. Mice typically produce immunoglobulins IgG1 or IgG2a. Specific assays have been developed to detect anti-AAV antibodies. Enzyme-linked immunosorbent assay (ELISA) allows fast and accurate detection of various inhibitory and not inhibitory antibodies.

[5]Coagulation of plasma samples from hemophilic mice is determined by measuring aPPT, activated partial thromboplastin time using fibrometer. Range of aPPT in hemophilia B mice is 55–100 s, 25–30 s in normal mice.

[6]Blood collection in mice can be performed via tail or retro-orbital bleeding. Mice need to be anesthetized for blood collection. Retro-orbital bleeding yields 50–150 μl of blood collected in to heparinized glass capillaries and can be used for ELISA assays. For Bethesda and APPT, tail bleed is a preferred method; blood is collected into citrate or oxalate containing tubes.

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hemophilia B (*see* MASAC Recommendations #137 and #160, http://www.hemophilia.org/research/masac/masac_all.htm).

2 Materials

2.1 Rodent Surgery

Rodent survival surgeries do not require a separate facility. Surgeries can be performed in a regular procedure room in the areas that can be easily sanitized. All instruments used for surgery should be sterilized by autoclaving 270 °F for 10 min prior to use. For multiple surgeries during single session, instruments must be disinfected between animals using hot bead sterilizer.

2.1.1 Instrument Kit

1. 2 scissors.
2. 2 retractors.
3. Forceps.
4. 1 ml syringe.
5. 30Ga needles.
6. Sterile Q-tips.
7. Sterile gauze.
8. Sterile, absorbable haemostatic material.

2.1.2 Additional Equipment

1. Animal clippers, blade#40.
2. Anesthesia machine for rodents, e.g., SurgiVet.
3. Isoflurane, oxygen tank.
4. Purelube, ophthalmic ointment.
5. Sterile PBS.
6. Water-recirculating heating pad.
7. Sterile surgical gloves, gown, and face mask.

2.2 Canine Surgery

In contrast to rodents, survival surgeries in dogs require a separate dedicated surgical facility that meets or exceeds requirements for performing survival surgery. All instruments used for surgery must be sterilized by autoclaving using standardized protocols.

2.2.1 Standard “Vet Pack” Instrument Kit for Major Abdominal Surgery

1. Vet Pack (abdominal pack).
2. 4 towel clamps.

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3. 1 operating scissors.
4. 1 curved mayo scissors.
5. 1 curved Metzenbaum scissors.
6. 1 thumb forceps with teeth.
7. 1 thumb forceps without teeth.
8. 1 needle holder.
9. 4 curved mosquito forceps.
10. 4 straight mosquito forceps.
11. 4 curved Kelly forceps.
12. 4 Rochester-Carmalt forceps.
13. 1 Haight Rib Spreader.
14. 2 #3 blade handles.
15. 2 Allis tissue forceps.
16. 2 Babcocks.
17. 2 Peans.
18. 1 small suction tip.
19. 1 large suction tip.
20. 1 spay hook.
21. 8 surgical towels.
22. 3 × 3 gauze squares.
23. 1 female catheter.
24. 1 sponge forceps.
25. 1 stainless steel bowl.
26. 1 Weitlaner.
27. 1 catheter introducer.

2.2.2 Additional Equipment

1. Animal clippers.
2. Anesthesia machine for larger animals, e.g., SurgiVet.
3. Isoflurane, oxygen tank.
4. Ophthalmic ointment.
5. Sterile PBS.

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6. Heating pad.
7. Sterile surgical gloves, gowns, and appropriate face mask.

3 Methods

3.1 Rodent Surgery

3.1.1 Preparation of the Surgical Area

1. Surgical area must be disinfected prior to the surgery using any of the approved (appropriate) disinfectants in your facility.
2. Heating pad should be sanitized and placed on the cleaned surgical area.
3. Place sterile surgical wrap on the disinfected surface of the water-recirculating heating pad.
4. Open sterile instruments, gauze, Q-tips on the sterile surgical wrap.

3.1.2 Preparation of the Animal

1. For hemophilia B mice administer 200 μl of normal mouse plasma IV via tail vein 30 min before the surgery (*see* Notes 7 and [8]).
2. Place mouse into induction chamber of the anesthesia machine and administer via inhalation 5 % of isoflurane in 21 % oxygen carrier (flow of 2 L/min).
3. Once unconscious the mouse is removed from the chamber, abdomen quickly shaved from xiphoid down to the groin, purelube is applied to the both eyes to prevent corneal drying and placed on its back with the face inside the nose cone part of anesthesia equipment in the non-sterile area.
4. Clean and aseptically prepare surgical site using an appropriate scrubbing technique: starting in the middle, going in circle, gradually enlarging circular pattern. Use three alternating scrubs of Betadine solution and 70 % ethanol.
5. Move the animal to the clean surgical area and place on top of a heating pad to minimize hypothermia.
6. Use sterile scissors to cut a small hole in the sterile drape and cover animal with the drape only exposing abdomen.

3.1.3 Preparation of the Surgeon

1. Rodent surgeries do not require sterile gowning. Surgeon must wear clean lab. coat, face mask, hair cover, and sterile surgical gloves.

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[7] In this chapter, hepatic gene transfer to hemophilic mice is described. The identical procedure can also be performed in hemostatically normal mice to measure gene transfer or correct other disorders. Non-hemophilic mice will not require presurgical administration of normal mouse plasma.

[8] Vector doses of 10^{10}–10^{11} vg/mouse are typical for AAV2 and AAV5 expressing FIX. Higher doses may be required for FVIII expression in hemophilia A mice. Other serotypes or improvements in vector design (capsid, expression cassette, and so on) may allow for reduced vector doses.

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3.2 Surgical Procedure

1. Once animal has been shaved, scrubbed, and moved to the sterile surgical area and covered with the sterile surgical drape while exposing abdomen, make a small (<1 in.) skin incision from the bladder up to the level of the xiphoid.
2. Repeat same opening technique for the muscle layer.
3. Retract the skin and muscle layers on both the right and left sides. Place tissue retractors to hold them in place.
4. Place a piece of sterile gauze over the left retractor and saturate gauze with the sterile PBS.
5. Using a sterile Q-tip, carefully move intestines onto the gauze and roll pancreas over to expose the portal vein (Fig. 1a). Portal vein is located on the ventral (bottom) part of the pancreas.
6. Once portal vein is exposed, keep applying slight traction with the sterile Q-tip on the pancreas near the vein bed to create tissue tension for the insertion of the needle (Fig. 1b).
7. Begin inserting needle into the body of the pancreas ~2 mm below the vein, keep advancing it into the vein until needle tip will become visible through the vein wall (Fig. 1c). Slowly depress the plunger and release the vector solution into the vein (Fig. 1d).
8. Using sterile dry Q-tip, apply pressure next to the needle insertion place, slowly retracting the needle and rolling Q-tip to cover the hole from the needle to prevent backflow of blood from the vein.
9. Hold pressure on the vein for at least 30 s. Cut a small piece of absorbable haemostatic material and place on injection site before replacing intestines back into the abdomen and prepare for suturing.
10. Close the abdominal wall using 4-0 silk, non-cutting taper point or round needle, continuous pattern. Place second layer of sutures on the skin, using Ethilon 4-0 suture material with cutting-edge needle and interrupted suture pattern.
11. Disconnect anesthesia and administer analgesic caprofen at 5 mg/kg or buprenorphine at 0.05–0.1 mg/kg.
12. Postsurgical care: Mice are kept on the heating pad for the entire duration of the surgery and postsurgical recovery to avoid hypothermia. Administration of normal mouse plasma (200 μl) is repeated approximately 30 min after surgery. Animals should be visually inspected frequently for bleeding, wound healing, and possible infection in the first 48 h and daily after that.
13. Sutures should be removed 10–14 days after surgery.

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3.3 Canine Surgery

3.3.1 Preparation of the Surgical Area

1. Surgical area must be disinfected prior to the surgery using any of the approved (appropriate) disinfectants in your facility.
2. Heating pad should be sanitized and placed on the cleaned surgical area.
3. All personnel must wear surgical clothing, hair cover, face mask, shoe covers, and, if in contact with the sterile field, sterile surgical gloves.

3.3.2 Preparation of the Animal

1. General anesthesia: Typically, dogs are premedicated with Atropine SQ (0.06 mg/kg) and then induced with propofol (“Propoflo28” at 3.2 mg/kg to 7.6 mg/kg IV over 60–90 s) followed by immediate intubation and transition to isoflurane to effect (~2 %). Nitrous oxide (50 %) is occasionally used during induction. Anesthesia is evaluated by direct observation of heart rate, respiratory rate, blood pressure, oxygen saturation, end-tidal CO_2, body temperature, and persistence or absence of palpebral, corneal, and withdrawal reflexes. A computer-based system and hand-written notes are used to record events that occur perioperatively.
2. Once the dog is anesthetized and intubated, ophthalmic ointment is applied to both eyes to prevent corneal drying.
3. Fur is shaved from the surgical field, generally from the lower rib cage to the lower abdomen. The shaved skin is cleaned with Betadine solution and 70 % ethanol. The dog is covered with sterile surgical drapes.

3.3.3 Surgeon Preparation

1. Survival surgeries in dogs require surgical hand scrubbing and sterile gowning and gloving. The surgeon must wear shoe covers, a face mask, and hair cover as do all of the operation room attendees and assistants.

3.4 Surgical Procedure

1. Prior to making an incision, canine FIX levels are raised to at least 10–20 % by infusing an appropriate amount of normal canine plasma. One needs to know the weight and hematocrit of the dog. The total blood volume is estimated at 40 ml/lb (18.18 ml/kg).
2. Therefore,

$$\begin{aligned}&\text{Wt (kg)} \times 18.18\,\text{ml/kg} = \text{Total Blood Volume (TBV)}\\&100 - \text{Hematocrit} = \%\text{Plasma Volume}\\&\text{TBV} \times \%\,\text{Plasma Volume} = \text{Total Plasma Vol in ml}\end{aligned}$$

Combined equation: (wt kg × 18.18 ml/kg) × (100 − hematocrit) = Total Plasma Volume in ml.

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Then, to raise FIX levels to 10 % in a dog with 900 cc calculated plasma volume, 100 cc of normal plasma is infused with FIX at 1 unit/ml.

Additional normal plasma can be given if the surgeon feels hemostasis is inadequate. The whole blood clotting time can be given a quick estimate of whether or not FIX levels are above 5 % [35]. Meticulous attention must be paid to controlling bleeding as incisions are made. There is often a subcutaneous vein of substantial size just beneath the skin in the midline incision site that requires attention.

3. A skin incision is made from the xiphoid down to the lower abdomen (~10 cm) and then the abdominal cavity is opened via its muscle layers.

4. A baseline liver biopsy is taken for use as control tissue if a follow up biopsy is performed to determine vector copy number, gene expression or if there is cellular toxicity. A small wedge of liver tissue is encircled with suture material, and the tissue is processed for molecular and histological studies.

5. The spleen is carefully exteriorized (Fig. 2, panel 1), and a 3–5 French balloon-tipped catheter is prepared by inflating the balloon to check for leaks (~0.5–1.0 cc, Fig. 2, panel 2).

6. An appropriately sized branch of the splenic vein is isolated, and circumferential sutures are positioned proximally and distally over about 2 cm; this branch of the splenic vein is punctured and the balloon-tipped catheter is inserted and advanced into the portal vein, usually between 10 and 20 cm (Fig. 2, panels 2–5). The position of the catheter in the portal vein is confirmed visually or by palpation.

7. The distal port of the balloon-tipped catheter is aspirated to confirm blood can be withdrawn even when the balloon is securely inflate and occluding the portal vein. This may require a “trial and error” approach until positioning is optimized.

8. When the catheter position is optimized, a small amount (~1 cc) of vector is infused while the balloon is inflated. Infusion rates are adjusted as the blood pressure tolerates. In general, AAV vectors are well tolerated and can be infused over 15 or 30 min.

9. After the vector is infused, the catheter is removed, the branch of the splenic vein is ligated proximally and distally, and the spleen is returned to the abdomen being careful not to injure it (Fig. 2, panel 6).

10. The surgical incision is closed in layers using suture material (Fig. 2, panels 7 and 8). Each layer is carefully examined for bleeding sites. Extra time spent achieving hemostasis at this point may prevent reoperation for small but significant bleeders.

11. Discontinue anesthesia and extubate when appropriate gag reflex is documented. Care must be taken to avoid trauma to the airway that can result in hemorrhage and airway obstruction.

12. The dog receives an intramuscular injection of procaine penicillin (or other prophylactic antibiotic as prescribed by the attending veterinarian) alone with

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postoperative analgesia and is allowed to recover under observation. The dog and its incision site are observed daily, and immediate steps are taken to treat infection or bleeding as is appropriate. In general, FIX is given daily for 7 days postoperatively or longer if needed. The exogenous plasma FIX disappears to undetectable levels (<0.1 %) within 21–28 days [35].

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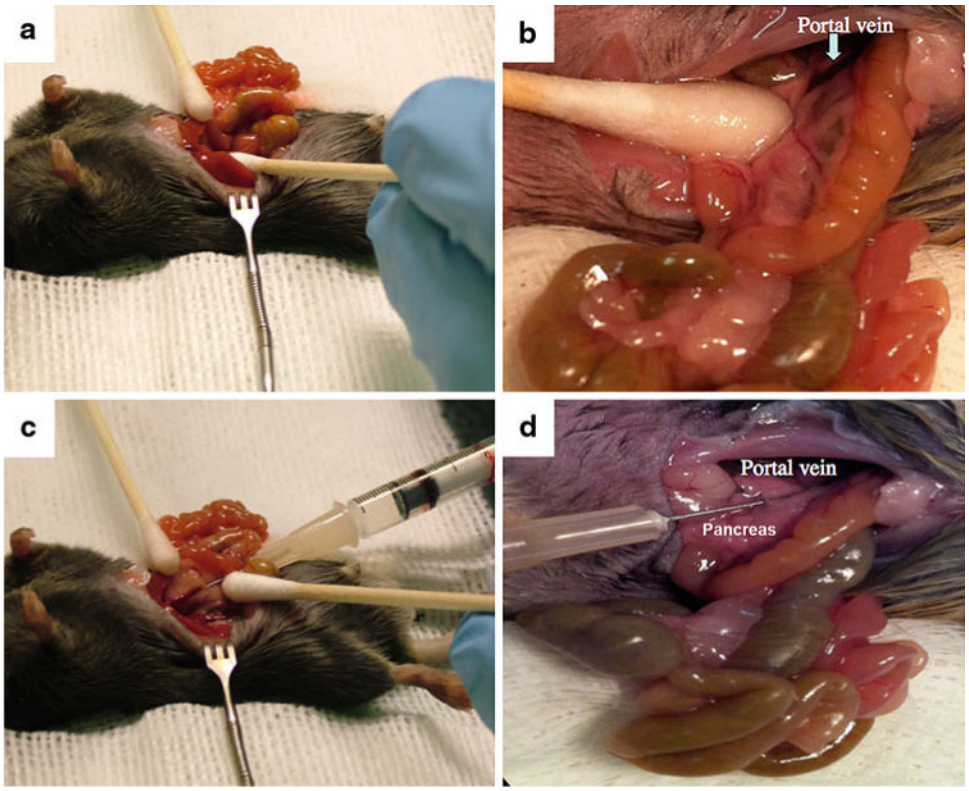

Fig. 1.

Portal vein delivery of gene therapy vector to a mouse. (**a**) Displacement of intestinal tract and pancreas to gain access to portal vein. (**b**) Exposure of portal vein. The area directly caudal to the portal vein must be flat enough to allow a needle to enter the vessel without inhibiting the syringe. A Q-tip is used to “retract” the pancreas to put tension on the vessel. (**c**) Insertion of needle through the multiple layers to enter the portal vein. One should see the bevel of the needle in the vessel. (**d**) Vector administration. Blanching of the liver should be obvious when depressing the plunger while observing the liver

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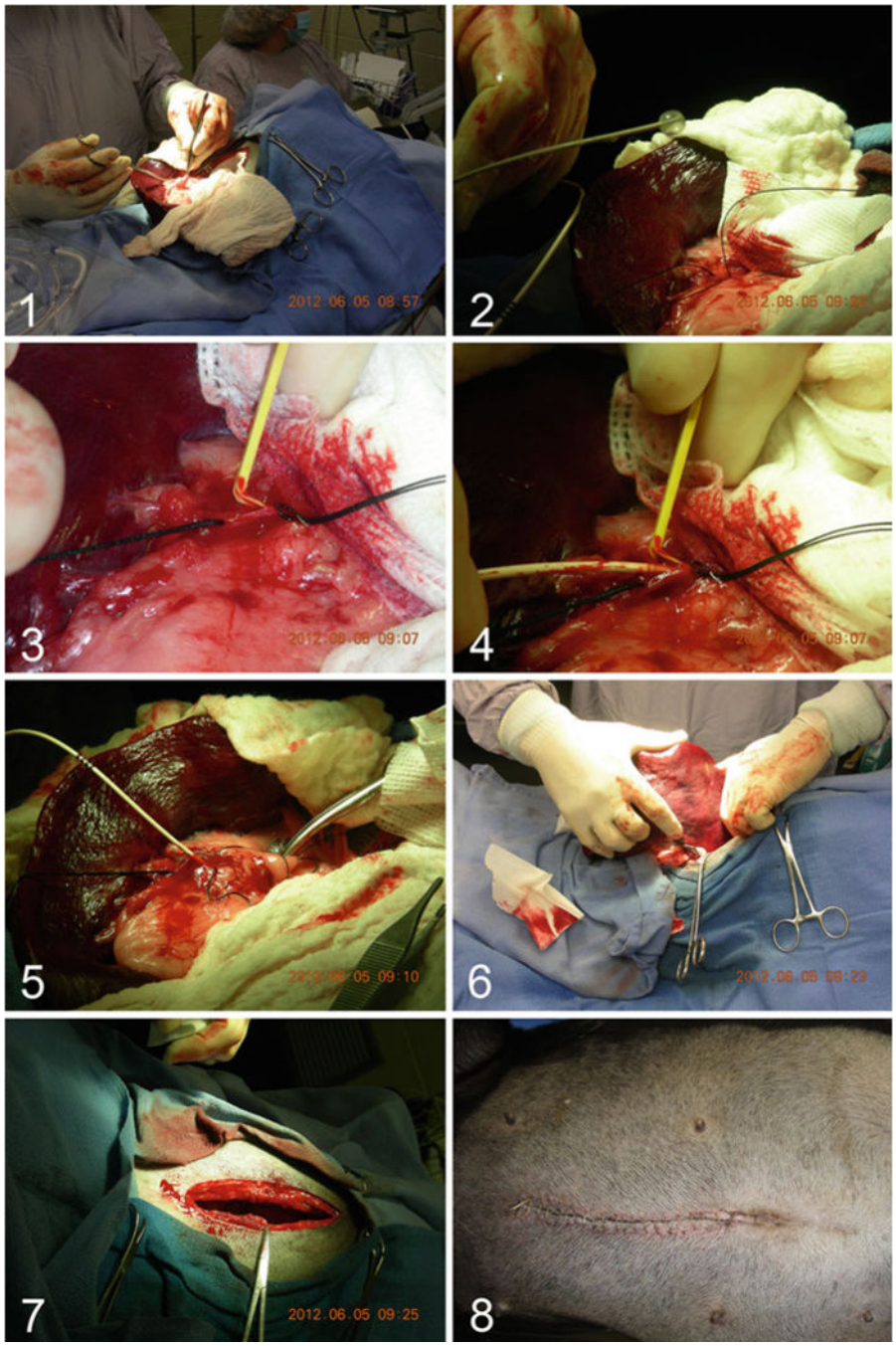

Fig. 2.

Portal vein delivery of gene therapy vector to a hemophilic dog. *Panel 1*: The spleen is exteriorized via a midline laparotomy incision. *Panel 2*: A 3–5 French balloon-tipped catheter is prepared by inflating the balloon to check for leaks. *Panel 3*: A small splenic vein is isolated and a venotomy is performed. *Panel 4*: The balloon-tipped catheter is inserted in the venotomy site and passed antegrade to the hepatic portal vein, usually 10–20 cm. *Panel 5*: With the balloon inflated, the gene therapy vector is administered to the liver. *Panel 6*:

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The spleen is returned to the abdomen. *Panels 7 and 8*: The laparotomy incision is repaired with careful attention to bleeding vessels