Intraarticular factor IX protein or gene replacement protects against development of hemophilic synovitis in the absence of circulating factor IX

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Hemophilic bleeding into joints causes synovial and microvascular proliferation and inflammation (hemophilic synovitis) that contribute to end-stage joint degeneration (hemophilic arthropathy), the major morbidity of hemophilia. New therapies are needed for joint deterioration that progresses despite standard intravenous (IV) clotting factor replacement. To test whether factor IX within the joint space can protect joints from hemophilic synovitis, we established a hemophilia B mouse model of synovitis. Factor IX knockout ($FIX^{-/-}$) mice received a puncture of the knee joint capsule with a needle to induce hemarthrosis; human factor IX (hFIX) was either injected through the needle into the joint space (intraarticularly) or immediately delivered IV. $FIX^{-/-}$ mice receiving intraarticular FIX protein were protected from synovitis compared with mice receiving same or greater doses of hFIX IV. Next, adeno-associated virus (AAV) gene transfer vectors expressing hFIX were injected into knee joints of $FIX^{-/-}$ mice. Joints treated with 10^{10} vector genomes (vg)/joint AAV2-, AAV5-, or AAV8-hFIX or 2.5×10^9 vg/joint AAV5-hFIX developed significantly fewer pathologic changes 2 weeks after injury compared with the pathology of control injured contralateral hind limbs. Extravascular factor activity and joint-directed gene transfer may ameliorate hemophilic joint destruction, even in the absence of circulating FIX. (Blood. 2008;112:4532-4541)

Introduction

The most significant morbidity resulting from congenitally deficient factor VIII or IX activity (hemophilia A or hemophilia B) is the progressive destruction of joints resulting from recurrent intraarticular (IA) hemorrhage. Although bleeding at other sites does occur in persons with hemophilia, the musculoskeletal system is by far the most common site; 85% of all bleeding events occur in joints, and 80% of these affect 6 problem joints: the elbows, knees, and ankles.[1] Joint hemorrhage is treated by intravenous (IV) infusion of clotting factor to raise the circulating plasma activity. There is a need for adjunctive therapies directed specifically to the pathology within the hemophilic joint.

An understanding of the pathophysiology of hemophilic joint disease is only now emerging.[2-4] Joint bleeding results in a chronic inflammatory disorder known as hemophilic synovitis, which in time evolves into a complex arthritis termed hemophilic arthropathy, in which the synovial disease is accompanied by degenerative changes in cartilage and underlying bone.[3,4] As the inflammatory environment that develops in response to blood in a joint stimulates neoangiogenesis of fragile blood vessels, one or more "target" joints for recurrent bleeding develop. Joint-surface erosions secondary to chronic synovitis often occur in early childhood.[5] If aggressive early prophylactic factor replacement is not instituted, 90% of persons with severe hemophilia (< 1% factor VIII or factor IX activity) will have chronic degenerative changes in 1 to 6 joints by 25 years of age.

A limited number of treatment options exist for recurrent joint bleeding and hemophilic synovitis.[6] The mainstay of therapy is replacement clotting factor dosed to achieve a circulating plasma activity level adequate to provide hemostasis throughout the body. Factor replacement in response to ongoing bleeding does not halt the progression of existing arthropathy.[5] Instead, institution of uninterrupted preventive (prophylactic) factor infusions at an early age, before the onset of recurrent joint bleeding, should be the standard of care.[7] The major costs of hemophilia to the healthcare system in dollars, to society in lost productivity and to the person with hemophilia in terms of quality of life, result from bleeding into joints.[8]

For persons who bleed despite systemic factor replacement, adjunctive medical management is supportive and not specific to the pathology of the hemophilic joint, for example, systemic anti-inflammatory treatment (regular narcotics, COX-2 inhibitors, intermittent systemic corticosteroids), immobilization, and physical therapy. Surgical options include ablation of the end-stage joint (joint fusion or prosthetic replacement).[2,9,10] There are, however, circumstances when direct local therapy to the hemophilic joint is performed. Some experienced clinicians recommend needle aspiration of the fresh blood from acutely distended and ballottable hemarthrosis; nevertheless, concurrent IA injection of clotting factor has not been recommended.[2,9,10] For hemophilic joint bleeding that is resistant to conservative medical therapy, IA corticosteroids, hyaluronic acid, and chemical or radioactive compounds are examples of treatment injected directly into the joint, rather than attempts to correct the systemic coagulation system.[9,11-14]

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In recent years, gene therapy using adeno-associated virus (AAV) has been explored for the treatment of hemophilia, including hemophilia B clinical trials targeting muscle or liver.[15,16] At the doses used (up to 2×10^{12} vector genomes [vg]/kg body weight into the liver) persistent hemostatic factor IX activity has not been achieved in human subjects. Compared with systemic gene therapy, IA gene delivery may provide concentrated levels of the therapeutic protein within the joint, at the site of greatest risk of bleeding. The feasibility of IA gene therapy has been established by several groups who have used AAV to express genes in animal models of rheumatoid arthritis (RA) and osteoarthritis (OA).[17-21] Nevertheless, it is unknown whether directed replacement of clotting factor in synovial fluid or joint tissue can provide meaningful hemostatic protection compared with intravascular replacement.

Our investigations establish that IA factor IX delivered percutaneously or via AAV-mediated transduction of joint tissues decreases the progression of hemorrhage-related joint degeneration.

Methods

AAV vector constructs and production

The AAV vector containing the 1.4-kb hFIX cDNA (rAAV-CBA-hFIX) has been described previously.[22] All vectors were produced and titered at the UNC Virus Vector Core Facility as described previously.[23] Multiple serotypes of self-complementary AAV (scAAV) carrying green fluorescent protein transgene driven by the cytomegalovirus enhancer/chicken β-actin promoter were provided generously by Dr Zhijian Wu, and AAV-expressing firefly luciferase was provided kindly by Dr Joseph Rabinowitz (Thomas Jefferson University, Philadelphia, PA). All AAV vectors used in the study are under transcriptional control of cytomegalovirus enhancer/chicken β-actin promoter.[24]

Animal care and study

Wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Factor IX knockout ($FIX^{-/-}$) mice[22,25] were bred in house. All investigations were approved by the University of North Carolina–Chapel Hill Institutional Animal Care and Use Committee. Mice were anesthetized using intraperitoneal 1.25% Avertin for all procedures. Knee joint IA bleeding challenge was performed using a Hamilton syringe with 30.5-G needle via a small (~ 0.5 mm) incision of the skin overlying the patella as described.[26] All blood samples were collected from the retro-orbital plexus into 1:9 parts 3.2% citrated sodium and stored at −80°C. The knee joints were collected by sectioning the femur and tibia 1 cm from the joint, fixed, and decalcified using routine histologic procedures.

In vivo administration of local or systemic factor IX protein therapy

Study groups of $FIX^{-/-}$ mice received recombinant human factor IX (rhFIX, BeneFix, Wyeth, Philadelphia, PA) at a range of doses injected either via tail vein (IV) or intraarticularly via a 30.5-gauge needle inserted into the left hind limb knee joint. Mice receiving IV factor IX then received a single puncture of the left hind knee joint capsule with a 30.5-gauge needle within 15 minutes of the IV dose and IA instillation of 5 μL of normal saline (NS), to reproduce the bleeding challenge experienced by the IA study groups. Fourteen days after injury, both knee joints were collected for histologic examination (≥ 3 animals in each treatment group). rhFIX doses for IA study groups were 20 IU/kg, 10 IU/kg, 5 IU/kg, or 2.5 IU/kg. Total volume for all doses was 5 μL. rhFIX doses for IV study groups were: 100 IU/kg, 50 IU/kg, and 25 IU/kg. A negative control group received 5 μL NS IA, without any rhFIX.

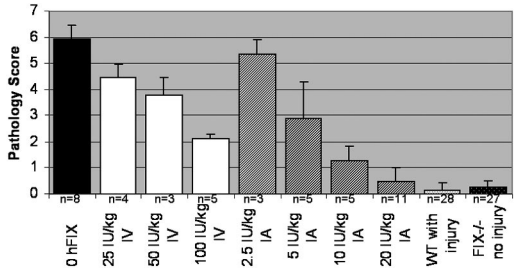

Figure 1. IA delivery of FIX affords protection from synovitis compared with systemic administration of FIX. $FIX^{-/-}$ mice received either IV recombinant hFIX followed by a joint capsular needle puncture injury and NS OR needle puncture with coincident IA injection of hFIX. A group of $FIX^{-/-}$ mice received no FIX and puncture with normal saline as a concurrently injured negative control (■). Fourteen days after the capsular puncture injury to left hind limb knee joint, left (injured) knee joints were collected for histologic examination using a validated mouse hemophilic synovitis grading system; uninjured (right) knee joints of the mice were also graded (far right column, "$FIX^{-/-}$ no injury"). A grade of zero to 10 is awarded for increasing pathology based on parameters of synovial hyperplasia (0-3 points), vascularity (0-3 points), or the presence of blood, synovial villus formation, discoloration by hemosiderin, or cartilage erosion (0 = absent; 1 = present). Average scores with the SEM are shown. Differences between IA treatments of 20 IU/kg and 10 IU/kg, compared with 100 IU/kg IV, were statistically significant ($P < .001$, $P < .05$, respectively).

Histologic grading of synovitis pathology

Hemophilic synovitis was graded according to a validated system, which awards 0 to 10 points for increasing evidence of synovial overgrowth, neovascularity, and articular bleeding (Figure 1).[27] Three or more independent reviewers blinded to the experimental conditions examined the entire joint space and articular surfaces of 2 adjacent sagittal sections from each knee. Areas of greatest synovial thickening and vascularity were identified, and 3 nonoverlapping fields from these regions were scored at high magnification and total synovitis scores from each knee averaged. Images were captured with a DMX-1200 color camera using the Act-1 software (Nikon, Melville, NY).[28]

Circulating FIX after IA versus IV delivery

To examine factor IX recovery, a single dose of 20 IU/kg IA or 20 IU/kg or 80 IU/kg IV was given and citrated plasma collected at 15 minutes, 1 hour, and 2 hours after injection to examine FIX recovery. To examine extended survival of FIX, both 25 IU/kg and 100 IU/kg hFIX doses were studied using IA or IV delivery. Plasma collected at 1, 4, 12, 24, 48, and 72 hours was studied in a one-stage factor IX activity assay.[22]

Detection and survival of FIX in synovial fluid after IA or IV delivery

A single dose of 100 IU/kg IA (in 5 μL total volume) or 100 IU/kg IV was given, and synovial fluid was collected at 1, 4, 12, 24, 48, and 72 hours from separate cohorts (n = 6-7 mice at each time point). At the designated time point, mice were killed, the skin overlying the treated knee was excised, the patellar ligament dissected, and a 30.5-G insulin syringe inserted into the joint capsule to instill 20 μL of NS. Saline wash and synovial fluid were immediately aspirated and saved, followed by an additional 20 μL of synovial lavage.[21,29] hFIX in the combined synovial lavage fluid was measured by enzyme-linked immunosorbent assay (ELISA), as previously described.[30]

In vivo administration of IA factor IX gene therapy

Hemostatically normal mice received AAV2, AAV5, or AAV8 expressing either firefly luciferase (for bioluminescence imaging, Figure 2) or human factor IX (for immunohistochemistry, Figure 3), and expression was assayed at the time points indicated. At the time of delivery of gene therapy IA, $FIX^{-/-}$ mice were given hemostatic protection using 100 IU/kg of hFIX IV, then were injected in the left knee with either lower-dose (2.5×10^9 vector genomes vg) or higher-dose (10^{10} vg) AAV2, AAV5, or AAV8 vectors in a total volume of 5 μL. The right knee received a capsular

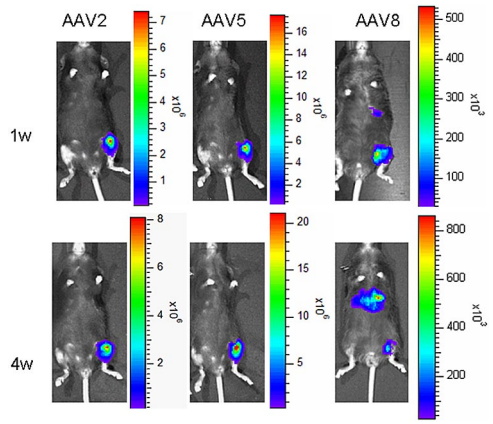

Figure 2. Comparison of localization of gene expression to joint using different serotypes of AAV: in vivo bioluminescence imaging. Wild-type mice received AAV. Luciferase vector packaged in capsid from serotypes AAV2, AAV5, and AAV8 at the dose of 8×10^8 vg/animal by IA injection to the left hind limb knee joint. Bioluminescence imaging with a CCD camera (IVIS, Xenogen) was initiated and recorded exactly 15 minutes after injection of D-luciferin, the substrate of luciferase. After acquiring a grayscale photograph, a bioluminescent image was captured with adjusted exposure time, binning (resolution) factor, 1/*f* stop and open filter to acquire maximum signal while avoiding a saturating image. (Top panel) One week after vector delivery. (Bottom panel) Four weeks after vector delivery.

puncture as well, and 5-μL NS injection as control. After 4 weeks, bilateral knee injury was induced by needle puncture. Two weeks later, joints were harvested and pathology was graded. A final group of $FIX^{-/-}$ mice were injected in the left knee with 1.0×10^{10} vg AAV5-hFIX, and synovial fluid was collected 2 weeks and 4 weeks later to assess local hFIX expression after gene transfer.

Bioluminescence imaging of mice

Under isoflurane anesthesia, mice were injected intraperitoneally with 150 μg/g D-luciferin in PBS. Bioluminescence imaging with a CCD camera (IVIS, Xenogen, Alameda, CA) was initiated exactly 15 minutes after injection. Signal intensities from regions of interest are expressed as total photon flux (photons/s per cm^2).[31]

FIX activity and anti-hFIX Bethesda inhibitor assay

One-stage factor IX activity assay (FIX-specific aPTT) and factor IX Bethesda inhibitor antibody assay were performed as previously described, using a START 4 Coagulation Analyzer (Diagnostica Stago, Asnières, France).[22]

Immunohistochemical localization of hFIX expressed after AAV-hFIX

Paraffin-embedded samples of joint tissue were sectioned at 5 μm and processed for hFIX immunostain as previously described,[30] using a polyclonal rabbit anti-hFIX (Dako North America, Carpinteria, CA) as the primary antibody and detection of the horseradish peroxidase (HRP)–conjugated secondary antibody after incubation with diaminobenzidine and counterstaining with hematoxylin. The total number of cells and number of HRP-positive cells in 6 representative fields were counted, and the percentage of positive cells calculated. Separately, a conformation-dependent monoclonal antibody, A-7 antibody, was used to recognize gamma-carboxylated hFIX (antibody generously provided by Darrel Stafford, University of North Carolina at Chapel Hill).[32] Before adding primary A-7 antibody, mouse blocking reagent (Biocare Medical, Concord, CA) was first used 1 hour at 25°C to block the endogenous mouse IgG; detection of the hFIX/A-7 monoclonal binding was with HRP-conjugated goat anti–mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Microscopy

Images were captured on a Nikon Microphot SA microscope equipped with 10×/0.30, 20×/0.50, and 40×/0.70 numeric aperture objective lenses. Photographs were taken with a DMX-1200 color camera using the ACT-1 software (all from Nikon Instruments, Melville, NY).

Statistical analysis

Quantitative data are presented as mean plus or minus SD. Two-tailed paired Student *t* test was performed with statistical software package (SAS, version 9.3). *P* values less than .05 were considered a statistically significant difference.

Results

Development of hemophilic synovitis model in hemophilia B mice

Bleeding into the joints of hemophilic mice leads to clinical and pathologic changes that closely resemble human hemophilic synovitis. Two of the authors (L.A.V. and N.H.) have previously reported the use of a blunt injury bleeding model in hemophilia A mice.[28] Similar to hemophilic joint disease followed clinically, the progression to clinically obvious joint disease in that original model could be quite variable, resulting after a single injury in

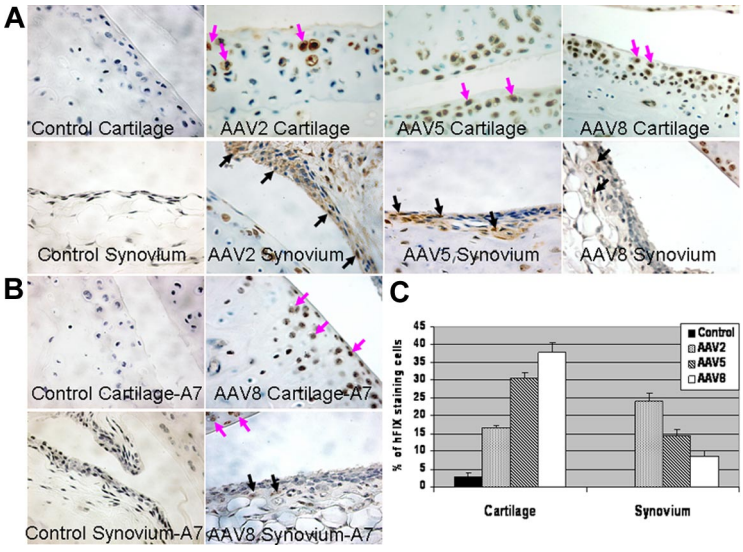

Figure 3. Immunohistochemical staining (IHC) of factor IX. Hemostatically normal C57BL/6 mice received IA injection of 2.5×10^9 vg AAV2, AAV5, or AAV8 expressing hFIX into the left knee joint capsule. Four weeks later, the AAV-treated knee and untreated control knee were collected, and immunostaining performed for hFIX using polyclonal anti-FIX (A) or using monoclonal antifactor IX A-7, specific for γ-carboxylated factor IX (B). HRP stain appears brown in FIX-expressing cells. Hematoxylin was added as counterstain in all images. (A) Control knee has primary (antifactor IX) antibody omitted and subsequent staining steps performed, demonstrating no background HRP (brown stain). Panels labeled AAV2, 5, and 8 cartilage demonstrate HRP staining cells within ssAAV2, 5, and 8-CBA-hFIX-transduced cartilage. Panels labeled AAV2, AAV5, or AAV8 synovium demonstrate HRP staining cells within ssAAV2, 5, and 8-CBA-hFIX-transduced synovium. (B) Immunostaining performed using monoclonal anti-FIX A-7 antibody. Control (negative) has primary A-7 antibody omitted and subsequent staining steps performed. Representative images of ssAAV8-CBA-hFIX transduced cartilage and ssAAV8-CBA-hFIX transduced synovium are shown after A7-HRP labeling. Pink arrows represent individual factor IX-staining chondrocytes within cartilage; black arrows, transduced FLS within the synovial lining (original magnification ×400). N = 3 to 5 animals per treatment group. (C) Quantitative analysis of cells in cartilage and synovium expressing hFIX after AAV2-, AAV5-, or AAV8-CBA-hFIX transduction. Data are represented as percentage of hFIX positive staining cells; error bars represent SD (n ≥ 4 animals/group).

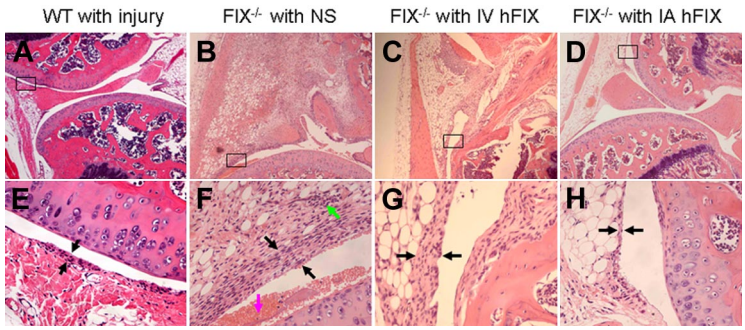

Figure 4. Representative histopathologic findings at 2 weeks after joint bleeding challenge. (A,E) Wild-type (WT, hemostatically normal) mouse knee joint after needle puncture and IA NS injection; joint space is well maintained with normal 3- or 4-cell layer synovial lining and no synovial hypervascularity. (B,F) FIX $^{-/-}$ mouse joint after needle puncture and IA NS injection; gross blood is seen in the joint space (pink arrow), which is narrowed by synovial proliferation (black arrow) with areas of discoloration resulting from hemosiderin staining (green arrow). (C,G) FIX $^{-/-}$ mouse joint after IV recombinant hFIX 50 IU/kg followed by needle puncture and IA NS injection; hypervascularity (low-power view) and synovial thickening (> 8-10 cell layers, black arrow, high-power view) are present. (D,H) FIX $^{-/-}$ mouse joint after needle puncture with coincident IA injection of hFIX 25 IU/kg; joint space is well maintained with thin synovial lining (black arrow) and smooth cartilage. Top panel, original magnification ×40; bottom panel, original magnification ×200.

some mice or after several bleeding challenges in others. For the studies of hemostatic protection that we proposed, a model that leads to reliable bleeding-induced pathology with each injury was desired, provided the ensuing pathology could be shown to occur in hemophilic but not hemostatically normal mice. A model of more severe blood-induced joint damage was characterized by the Valentino laboratory in factor VIII$^{-/-}$ mice, consisting of a single 30.5-gauge needle puncture of the knee joint capsule to induce hemarthrosis,[26] and was reported to induce similar pathology in factor IX$^{-/-}$ mice.[26,33] Because previous reports showed no data in factor IX$^{-/-}$ mice, we evaluated this model for the study of hemophilia B by validating the specificity of synovitis resulting from capsular puncture injury using large cohorts of wild-type mice (n = 28) and FIX$^{-/-}$ mice (n = 58). The single needle puncture does not cause arthropathy in hemostatically normal mice; the mean synovitis grade was 0.14 plus or minus 0.3, and no mice developed synovitis graded at more than or equal to 2 (additive pathology scale, 0-10). In contrast, the same injury results in synovitis (histopathology grade of ≥ 2) in more than 96% control hemophilia B mouse joints, with a mean synovitis grade of 4.4 plus or minus 2.0. Finally, the development of pathology is specific to the induced bleeding injury; uninjured joints of young adult factor IX$^{-/-}$ mice do not show significant synovitis (n = 27 joints examined, mean synovitis grade 0.26 ± 0.27).

IA delivery of FIX provides protection from bleeding-induced synovitis

To investigate the possibility that FIX present in the joint space can protect against blood-induced joint damage in the absence of circulating FIX, a range of doses of recombinant hFIX was given either IA or IV coincident with the capsular puncture injury. A group of hemophilia B mice received the identical joint capsular puncture and IA NS as a concurrently treated negative control group. As shown in Figures 1 and 4, IA hFIX afforded significant protection against blood-induced synovitis. Pathology score in mice treated with 5 IU/kg IA was equivalent or superior to those of mice treated with 50 IU/kg IV human factor IX. IA treatment at the highest dose (20 IU/kg) resulted in minimal synovitis (mean score, 0.3).

IA FIX does not increase circulating FIX activity

We considered the possibility that, through technical error or other mechanism, the IA hFIX might enter the circulation and effect joint protection via systemic plasma activity. The circulating pharmacokinetics of plasma FIX activity were examined after hFIX dosing via the tail vein or IA. We first determined that mouse FIX and human FIX are not interchangeable in plasma. An IV dose of hFIX concentrate that would be expected to fully correct a human (80-100 IU/kg of body weight) only partially corrected the FIX $^{-/-}$ mouse to 10% to 15% activity; this was the highest dose of hFIX used in any of the treatment groups shown in Figures 1 and 4 and Table 1. Although IA treatment at doses of 20 IU/kg or lower protected against blood-induced joint damage (Figures 1 and 4), IA treatment at this dose did not result in any detectable circulating FIX activity in the first 2 hours after treatment (Table 1).

Previous studies have shown that FIX can be given subcutaneously, intramuscularly, or intratracheally, and via each of these routes FIX will enter the circulation in a delayed fashion compared with IV delivery.[34,35] Therefore, an extended pharmacokinetic

Table 1. Factor IX survival in circulation

Mouse	Dose/route	15 minutes	1 hour	2 hours
Initial circulating FIX activity in mouse plasma after recombinant hFIX				
FIX$^{-/-}$	20 IU/kg IA	< 1%	< 1%	< 1%
FIX$^{-/-}$	20 IU/kg IV	1.95 ± 0.57	1.63 ± 0.68	< 1%
FIX$^{-/-}$	80 IU/kg IV	15.7 ± 5.88	11.5 ± 5.56	8.75 ± 3.46

Mouse	Dose/route	1 hour	4 hours	12 hours	24 hours	48 hours	72 hours
Extended circulating FIX activity in mouse plasma after recombinant hFIX							
FIX $^{-/-}$ (n = 6)	25 IU/kg IA	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%
FIX $^{-/-}$ (n = 7)	100 IU/kg IA	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%
FIX $^{-/-}$ (n = 8)	25 IU/kg IV	4.5 ± 0.9	2.1 ± 0.6	1.4 ± 0.4	1.0 ± 0.6	< 1%	< 1%
FIX $^{-/-}$ (n = 9)	100 IU/kg IV	17.3 ± 3.3	11.8 ± 4.8	6.4 ± 2.1	3.8 ± 1.8	2.7 ± 1.1	1.5 ± 0.4

FIX$^{-/-}$ mice were given recombinant hFIX by intravenous or intraarticular administration at the dose of 20 IU/kg or 80 IU/kg for initial factor IX activity recovery or 25 IU/kg or 100 IU/kg for extended activity recovery. Citrated plasma was collected for FIX activity assay (aPTT), and data are presented as percentage of normal human plasma activity.
IA indicates intraarticular; and IV, intravenous by tail vein.

Table 2. Fall-off of hFIX in synovial fluid after intraarticular administration

Route/tissue	1 hours	4 hours	12 hours	24 hours	48 hours	72 hours
Intraarticular SF	11 511 ± 1155	5509 ± 896	384 ± 81.8	116 ± 32.0	52.3 ± 10.0	26.1 ± 3.6
Intravenous SF	12.5 ± 2.1	6.42 ± 2.88	ND	ND	ND	ND
Intravenous plasma	2530 ± 268	1552 ± 138	584 ± 18.5	123 ± 24.2	52.2 ± 5.9	24.3 ± 1.8

$FIX^{-/-}$ mice were given recombinant hFIX by intravenous or intraarticular administration at the dose of 100 IU/kg, $n \geq 6$ at each time point. All samples are detected via hFIX specific ELISA, reported as ng FIX/mL. The normal circulating level of FIX protein in human plasma is 5000 ng/mL.

SF indicates synovial fluid; IA, intraarticular FIX delivery; IV, intravenous FIX delivery by tail vein; and ND, not detectable.

study was performed to rule out the possibility that FIX might enter the circulation from a local "depot" after IA injection. In a control group of mice treated IV, FIX activity decayed with a half-life of 7 to 12 hours, consistent with previous experience in our laboratory and others.[36] Even using a 4-fold higher IA hFIX dose than was used in the joint protection studies (Figure 1), no FIX activity was detected in plasma up to 72 hours after IA delivery (100 IU/kg IA; Table 1).

Kinetics of FIX concentration within the joint space after IA and IV delivery

As neither the initial concentrations nor the survival of factor IX within the synovial space after protein therapy is known, we examined synovial fluid levels of factor IX after delivery of 100 IU/kg of hFIX as either an IV or IA dose. As shown in Table 2, hFIX entering the synovial fluid after IV delivery was near the lower limits of detection of the ELISA assay even at the earliest time points, whereas the plasma terminal half-life is typical for what we observe in mice (~ 6-7 hours). The initial local concentration of factor IX in synovial fluid after IA delivery was strikingly higher than the plasma level achieved from the equivalent IV dosing; however, it appeared to fall off rapidly in the first 12 hours. In contrast, the terminal phase fall-off in the synovial fluid closely resembled the fall-off of the intravenously delivered circulating factor IX.

Differential gene expression from AAV serotypes examined using serial in vivo bioluminescence imaging

Initial in vitro transduction experiments in primary human fibroblast-like synoviocytes (FLSs) and cartilage explants were performed using AAV2-, AAV5-, and AAV8-expressing green fluorescent protein (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Although these studies were consistent with the general paradigm that AAV serotypes display different tropisms, even within cell types of a single tissue, in vitro transduction by AAV vectors is often poorly predictive of in vivo results. Therefore, the ability to localize in vivo expression using IA injection was studied using AAV vectors expressing firefly luciferase, a marker gene the expression of which could be followed serially in joints. Single-strand AAV luciferase encapsidated in serotypes AAV2, AAV5, or AAV8 was injected into the left knee joint of adult mice at a dose of 8×10^8 vg/animal. Intensity and biodistribution of luciferase expression were imaged weekly. AAV2, AAV5, and AAV8 luciferase vectors all transduced joints in vivo. Expression was primarily confined to the injected joint 1 week after infection, although some luciferase expression from the AAV8 vector could be seen in extraarticular sites at that time point. By 4 weeks after infection, the majority of signal from AAV8 vector came from the hepatosplenic region, at a time the other serotypes localized expression to the articular or periarticular space (Figure 2).

IA delivery of AAV-hFIX: biodistribution of expression within and outside the joint

Before subjecting hemophilic animals to an injury, we examined the relative biodistribution of factor IX expression from the AAV serotype vectors. AAV2-, AAV5-, and AAV8-CBA-hFIX vectors were injected into the knee joints of wild-type C57Bl/6 mice at the dose of 2.5×10^9 vg/animal. Four weeks later, knee joints were harvested and immunohistochemical staining for human factor IX performed. The anti-hFIX antibody does not cross-react with mouse FIX.[30] As shown in Figure 3A and C, patterns of serotype tropism were consistent with those seen in vitro (Figure S1): AAV5 transduced both FLSs and chondrocytes, whereas AAV2 demonstrated a bias toward FLS transduction and AAV8 toward chondrocyte transduction. In addition, a monoclonal anti-hFIX antibody A-7 was used to detect hFIX in samples from the AAV8-transduced joint. Factor IX binding by A-7 antibody depends on the factor IX Gla- domain having at least 9 or 10 carboxylated glutamic acid residues.[32] The same pattern of factor IX expression is seen as with the polyclonal anti-hFIX (chondrocyte-predominant from the AAV8-hFIX), suggesting that the joint tissue can perform factor IX gamma-carboxylation, a critical posttranslational modification of all vitamin K-dependent proteins. Separate groups of mice were also treated with the same dose of AAV2-, AAV5-, and AAV8-CBA-hFIX vectors and killed at 4 weeks after infection to examine the persistence of factor IX expression and vector genomes localized in the injected knee joint or spread to the liver via systemic spread. When AAV2, AAV5, or AAV8 vectors are delivered systemically, the liver is the principal site of persistent expression.[37] The expression of protein and persistence of vector genomes using AAV2 and AAV5 in the current report occurred primarily at the site of IA injection without spread to the liver (Figures 2, S2). In contrast, the majority of factor IX synthesis and vector genomes persist in the liver rather than the injected joint after AAV8-CBA-hFIX IA delivery (Figures 2, S2).

IA AAV-hFIX protects against development of hemophilic synovitis

Examined next was the potential for IA AAV-directed expression of hFIX to protect the joint from a subsequent blood-induced injury (capsular puncture). Under hemostatic protection of 100 IU/kg body weight with IV rhFIX, $FIX^{-/-}$ mice (4 mice /group) were injected in the left knee with either lower dose (2.5×10^9 vg) or higher dose (10^{10} vg) ssAAV2-, ssAAV5-, ssAAV8-CBA-hFIX vectors. These doses are equivalent to approximately 10^{11} vg/kg body weight (lower dose) and 4×10^{11} vg/kg (higher dose). After 4 weeks of vector expression, both knees were subjected to capsular puncture to induce hemarthrosis. Two weeks later, joints were harvested and histopathologic synovitis grading was performed, comparing the animal's treated hind limb to injured untreated control hind limb.

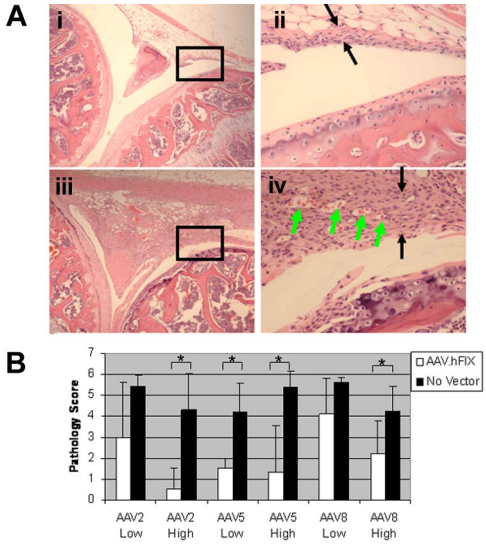

Figure 5. IA AAV-FIX expression within the joint protects from hemorrhage-induced synovitis. FIX $^{-/-}$ mice received AAV in left hind limb joint and normal saline in right hind limb joint under hemostatic coverage with IV FIX. Bilateral hind limb joints received puncture injury at 4 weeks after gene delivery; 2 weeks after injury, joints were collected. (A) Top panels: Representative pathologic changes of minimal synovitis at 2 weeks after puncture injury of knee receiving prior treatment with ssAAV5-CBA-hFIX (2.5×10^9 vg). Left, original magnification ×40; right (area of detail from left figure, indicated by box), original magnification ×200. Bottom panels: Injured untreated knee demonstrates changes of hemarthropathy, including synovial proliferation narrowing the joint space, synovial thickening (black arrows), foci of hypervascularity (green arrows), and synovial overgrowth of the tibial articular surface. Left, original magnification ×40; right (area of detail from left figure, indicated by box), original magnification ×200. (B) Histopathologic grading of mice treated with AAV-hFIX IA delivery and comparison with pathology score of contralateral injured untreated knee ("No vector" control). High dose, 10^{10} particles/animal; low dose, 2.5×10^9 particles/animal (n = 4 animals/group). *$P < .05$

After gene therapy, most mice in the higher dose AAV2- or AAV5-treated groups showed minimal synovitis; most joints scored 0 to 1 ($P < .01$ for the difference between injured contralateral control limbs and both ssAAV2-hFIX and ssAAV5-hFIX groups; Figure 5). There was considerable interanimal variability in most of the AAV-treated groups. ssAAV8-CBA-hFIX treatment at this dose also resulted in significant protection of treated joints (treated vs control knee, $P = .03$), although none of the mice treated with AAV8 showed complete protection (mean score, 2.22). The 4-fold lower dose of ssAAV2-CBA-hFIX and ssAAV8-CBA-hFIX did not demonstrate significant protection, but ssAAV5-CBA-hFIX at the lower dose was protective ($P = .03$).

The AAV2- and AAV5-treated animals did not have detectable circulating FIX at death (6 weeks after vector treatment) to account for the protective effect within the joint, as measured by ELISA or one-stage FIX activity assay. To examine this phenomenon, additional mice received AAV5-CBA-hFIX IA delivery and were killed for synovial fluid lavage after gene therapy; a low but relatively stable level of hFIX was detected in the dilute synovial fluid collected at 2 and 4 weeks after transduction (20.9 ± 4.7 ng/mL and 19.4 ± 8.6 ng/mL, respectively; n = 4 animals/time point). Low levels of FIX circulated in some AAV8-treated mice (10-300 ng/mL FIX antigen and < 1%-7.3% activity), consistent with the hepatic spread of AAV8 after IA virus demonstrated by the luciferase imaging. In addition, separate groups of mice treated IA with each vector were killed at 4 weeks after transduction, and the treated knee joint and liver were homogenized and assayed for hFIX protein by ELISA and hFIX transgene persistence. Minimal or no FIX was detected in liver compared with joint tissue treated with AAV2 or AAV5, corresponding closely to the distribution of hFIX genomes (Figure S2). In contrast, the majority of FIX was intrahepatic after AAV8 despite IA delivery, also corresponding with greater FIX genome persistence in liver (Figure S2).

Importantly, none of the mice developed anti-FIX antibodies detected by the Bethesda inhibitor assay during the 6 weeks after ssAAV-CBA-hFIX dose. This finding was unexpected because this strain of FIX$^{-/-}$ mice reliably develop neutralizing antihuman factor IX antibodies after intramuscular treatment with ssAAV2-CBA-hFIX vector.[22,38]

Discussion

The data presented in these studies suggest that directing factor replacement to the hemophilic joint could yield therapeutic benefits that augment standard systemic therapy. To perform these studies in FIX$^{-/-}$ mice required that we establish a mouse bleeding model in which a single joint injury reliably produces in the hemophilia B animal, but not in hemostatically normal animals, the joint pathology observed in human hemarthropathy. Under the conditions tested, this model shows, for the first time, that clotting factor IX within the joint space protects against the development of synovitis, in the absence of measurable circulating FIX activity or protein. Furthermore, these studies establish that persistent expression of FIX in the joint after AAV gene therapy may protect from hemophilic bleeding-induced joint pathology.

Rationale for an AAV gene therapy approach to hemophilic joint arthropathy

To our knowledge, there is no published experience using IA clotting factor replacement as a therapeutic strategy, probably because of (1) the general effectiveness of IV replacement therapy, at least in early joint disease; (2) a reluctance to potentially induce injury by putting a needle into a hemophilic joint; and (3) the reasonable assumption that the site of action for thrombin generation and hemostatic plug formation must be at the intraluminal surface of the injured vessel, not in extravascular tissues. Although aspiration of the acutely distended, painful hemophilic joint after systemic factor replacement (without IA clotting factor administration) is safe and can speed recovery after a severe joint hemorrhage,[2,39] repeatedly instrumenting the joints is not feasible, especially to prevent or interrupt early joint bleeding. The ideal gene therapy strategy would direct persistent replacement of the missing coagulation factor activity within the joint.

AAV2 transduction of FLS[18,20,21] and chondrocytes[17,19] has been demonstrated by others using multiple transgenes, including marker genes,[17,20] growth factors,[19] and cytokine inhibitors.[18,21] In vivo transgene expression for as long as 7 months has been demonstrated.[20] Many groups have chosen to examine transduction in only one of these cell types, eg, targeting synoviocytes to counter the inflammation of RA, as opposed to OA studies that target cartilage. Goater et al reported, as early as 2000, that a single AAV2 treatment could transduce both FLS and chondrocytes and that expression from AAV2 was more pronounced in the presence of joint damage than in normal joints.[40] A human phase 1 clinical trial of IA AAV2 expressing tumor necrosis factor-α receptor antagonist for RA is ongoing (www.clinicaltrials.gov as #NCT00126724). The safety of the IA AAV2 approach has received intensive investigation after the death of one subject on this trial. Postmortem tissue analyses demonstrated the transgene sequences were delivered relatively selectively to the target tissue of the study subject. The

subject was being treated concurrently with multiple systemic immunosuppressive drugs and died of disseminated histoplasmosis. The US National Institutes of Health Recombinant DNA Advisory Committee and the US Food and Drug Administration concluded that the vector was doubtful to have contributed significantly to the subject's clinical outcome (www4.od.nih.gov/oba/RAC/minutes/RAC_minutes_12-07.pdf).[41] Importantly, the IA gene therapy modeled in our report does not involve an immunosuppressive transgene.

AAV serotypes: observed differences in tropism and transduction after IA delivery

Alternative serotypes of AAV, compared with AAV2, have demonstrated divergent patterns of expression in multiple tissues and organs. Applications targeting synovium in rats and mice in vivo, which correlated with results in human RA cell lines in vitro, have suggested that AAV5 may direct higher expression than AAV2 in FLS.[42-44] AAV5 vectors have also demonstrated inflammation-inducible transgene expression.[44,45] Given the mixed proliferative, inflammatory, degenerative features of hemophilic arthropathy, we did not wish to restrict our target for expression to either FLS or chondrocytes exclusively; we thought it possible that therapeutic benefic might arise from expression in a variety of cell types in the joint or even combined expression from both IA and extraarticular sites. Our studies do not attempt a systematic examination of AAV serotype tropism in joint tissue. Based on experience in RA and OA applications, it was expected that AAV2 and AAV5 should transduce joints and, in particular, synoviocytes. This result was indeed observed in both our human joint tissue samples (Figure S1) and in mice in vivo (Figures 2, 3). The previously reported superiority of AAV5 compared with AAV2 for joint transduction was not demonstrated using the conditions of our experiment; of the serotypes we tested, AAV5-hFIX was, however, the only one that significantly reduced synovitis at the lowest dose (2.5×10^9 vg/animal).

The observed AAV8 tropism was not entirely expected. In our human tissue lines and explants as well as within the hemophilic mouse joint, the AAV8 vectors appeared to be relatively specific for chondrocytes and to show minimal expression in synoviocytes, consistent with the in vitro results reported by Boissier et al.[42] After IA delivery, AAV8 vectors displayed a natural tropism for the liver in both the luciferase and FIX expression systems. IA AAV8-hFIX produced low circulating FIX levels (from $< 1\%$ up to $\sim 7\%$ by one-stage factor IX activity assay). Despite this, the joint protection by AAV8-hFIX vector was not superior to that by AAV2 and AAV5 in this injury model. The results suggest that, after IA delivery of AAV-hFIX, the expressed clotting factor can concentrate within the joint space and the high localized factor levels provide joint protection that might augment or exceed the protection afforded by a lower level factor achieved by systemic gene therapy.

Extravascular clotting factor decreases development of blood-induced joint pathology: consideration of potential mechanisms and tissue-specific hemostasis

Little is known about the extravascular coagulation potential of normal synovial fluid, despite an extensive literature about coagulation parameters in inflammatory arthritis (ie, RA).[46-49] OA is considered a relatively noninflammatory arthropathy, and OA synovial fluid coagulation factors have been measured.[47,49] Procoagulant proteins of smaller molecular weight do have significant measurable activity in OA synovial fluid (eg, factor IX 10%, factor II 21% activity), whereas very large coagulants (factor V, VIII) are present at less than 1% of plasma activity. Chang et al reported that thrombin can be generated extravascularly in OA synovial fluid and that thrombin-generating potential falls off more rapidly in synovial fluid than in plasma.[50] These authors also noted that adding factor VIII or factor V in vitro rapidly enhances thrombin generation in OA synovial fluid; adding FIX was not tested in their system.

Although we demonstrate that factor IX instillation or endogenous expression within the joint protects the joint from the development of synovitis and blood-induced joint damage, it must be acknowledged that these studies do not allow conclusions regarding the mechanism for this protection. It can be speculated that the presence of a local concentration of FIX supports initial hemostasis of the injured joint vasculature. Although no systemic FIX activity was detectable, it is possible that FIX could enter the injured vascular space from the synovial (extravascular) side to contribute to thrombin generation at the intraluminal primary platelet thrombus. Alternatively, the potential for thrombin generation within synovial fluid has been demonstrated in vitro.[50] To assume that true extravascular coagulation within the synovial space tamponades or somehow staunches the bleeding from the injured vessel, current dogma predicts that phospholipid surface would be required to form a factor Xase complex; this conceivably could be provided by platelets that enter from the injured vessel.

Another possible mechanism of protection from synovitis would result if the IA factor maintains the stability of the initial hemostatic clot, thereby protecting from pathologic rebleeding. This would be possible if, for instance, the local survival of synovial FIX were prolonged. Using a cutaneous wound model in hemophilia B mice, McDonald et al have shown that, if hemostatic thrombin is generated only at the time of wounding (eg, a single IV dose of FIX that stops bleeding), wound closure is delayed compared with treatment continued for days after wounding. Wounds of $FIX^{-/-}$ mice receiving a single IV FIX dose showed greater and more prolonged iron deposition and macrophage infiltration than wounds of wild-type mice.[51] Keeping the cutaneous wound study in mind, our detection of factor IX measured in the synovial fluid for at least 72 hours after IA delivery is intriguing (Table 2). There is an initial rapid fall-off of IA factor IX, consistent with the in vitro observation of Chang et al of a rapid fall-off of thrombin-generating potential in synovial fluid compared with plasma.[50] This initial (α-) phase of factor IX consumption is probably influenced by collection in the setting of hemorrhage in our model. There follows a prolonged terminal (β-) half-life of the surviving synovial fluid factor IX, which indeed differs little from the terminal kinetics of circulating factor IX in the study group treated intravenously with the same FIX dose. We further documented low and steady levels of synovial fluid factor IX at 2 and 4 weeks after gene therapy ($< 1\%$ of the normal human factor IX level of ~ 5000 ng/mL of plasma) in the additional group of mice treated with IA AAV5-hFIX. The joint lavage method we used certainly dilutes the samples so that an estimation of minimal protective synovial factor IX levels cannot be reached using the methods of the current study. Nevertheless, the thrombin-generating or antifibrinolytic potential of this surviving synovial factor IX to prevent rebleeding (akin to IV prophylactic factor IX) warrants further investigation.

FIX survival in the synovial fluid might not be the only mechanism for sequestration of FIX within this tissue. The synovium of joints is rich in collagen IV,[52] an endothelial extracellular matrix protein to which factor IX binds specifically.[53] There is

rich vascular tissue factor (TF) expression in sites, such as cardiac muscle and the central nervous system (sites in which even minimal bleeding may be life threatening), potentially allowing rapid TF/VIIa pathway initiation, described as "tissue specific hemostasis."[54] The uninjured joint tissues and fluid have minimal vascular TF expression.[54,55] Therefore, it is possible that sequestration of intrinsic pathway factor IX in this site (eg, via collagen IV interaction) is a different example of tissue specific hemostasis, which protects nonhemophilic joints from hemarthropathy.

A final consideration is that IA FIX could interrupt some mediator of inflammation that promotes the inflammatory changes in the hemophilic joint. Functional TF activity is increased and tissue factor pathway inhibitor decreased in inflammatory arthritis (RA) compared with noninflammatory arthritis (OA). TF/VIIa activity is implicated in generating the thrombin and fibrin deposition that complicates RA and also in stimulation of vascular smooth muscle cell migration,[46] for example, via TF/VIIa activation of Protease Activated Receptor (PAR) 2.[56] Thrombin in turn is implicated in synovial thickening[48] and proteolysis of cartilage,[57] and these processes are also central to hemophilic synovitis. To carry the example further, thrombin activates PAR 1, which can up-regulate both pro- and anti-inflammatory chemokines.[58,59] One might speculate that, in the absence of intrinsic pathway proteins, the regulation of the TF/VIIa/tissue factor pathway inhibitor system and thrombin generation is unbalanced in a way that promotes hemophilic synovitis independently of initial hemostasis, and the balance is restored by supplying IA FIX. As an example, after the cutaneous wound injury, $FIX^{-/-}$ mice down-regulate perivascular TF expression, and do so longer and more profoundly than do hemostatically normal mice.[60] We measured quite high initial synovial levels of factor IX after IA protein delivery to the joint. There is no evidence to suggest whether there may be risks resulting from these initially very high levels that will counter the demonstrated benefit (avoiding subacute hemophilic synovitis). Our ongoing studies evaluate this injury model and the effect of the IA treatment approach on chronic hemophilic joint pathology.

Hemophilic animal models to establish pathophysiology of bleeding-induced joint damage and joint-directed therapy

The joints of our hemophilia B mice did not have preexisting arthropathy before protein or gene therapy. We are currently adapting and validating the injury model to reproduce the clinical scenario of advanced hemophilic arthropathy so as to confirm the efficacy of local factor protein or gene therapy in damaged joints. Care will need to be taken to validate a chronic arthropathy histology scale that quantitates additional endpoints for the cartilage and bone damage prevalent in chronic disease but not incorporated in the synovitis scale used in this report. After validating such a tool, the efficacy of IA hemostasis in long-standing hemarthropathy deserves evaluation, as the current study can be considered to model preventive therapies in joints without preexisting disease. Several groups have now reported that inflammatory cytokines stimulated by underlying joint disease actually can increase transgene expression from AAV2 vectors.[18,40,61] Nevertheless, persistence of episomal transgene expression from chronically damaged joint tissues may well be limiting and requires study. In addition, the novel presentation of potential alloantigens (eg, factor IX protein or vector capsid proteins) in the setting of a chronically inflamed joint could conceivably stimulate immune responses against transduced cells that were not seen in our experiments in naive joints.[62]

Modeling bleeding-induced pathology in hemostatically normal animals (eg, with autologous blood challenge), compared with the hemophilic joint injury reported here or the recently reported cutaneous wound model, does not accurately reveal pathology and potential therapeutic approaches. The mouse models also enable studies with cohort sizes that cannot practically be accomplished using hemophilic canines. Confirmation of efficacy of the IA approaches in large animals will be valuable, nonetheless; the progression of hemarthropathy may depend on weight-loading of the bleeding-damaged joint, with the effect increased in heavier animals.[63] In addition, gene expression using AAV serotypes has not always translated directly from mouse to large animals.[64]

These investigations, documenting the capacity of IA FIX protein or gene therapy to protect the joint from bleeding-induced synovitis, suggest that joint-directed therapies may be useful adjuncts in hemophilia care, in addition to the intravascular replacement of deficient factor activity. In addition, the studies underscore knowledge deficits regarding tissue specific hemostasis as well as the potential intersections of coagulant proteins and inflammatory mediators within the hemophilic joint that may be explored in this model.

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Authorship

Contribution: J.S. designed, executed, and interpreted all experiments and wrote the manuscript; N.H. designed experiments, including development of the joint injury model, scored the pathology, and reviewed the manuscript; L.A.V. contributed intellectual advice, including development of the joint injury model, scored the pathology, and edited the manuscript; R.J.S. contributed viral vectors as well as intellectual advice and edited the manuscript; B.L.F. modeled and performed the statistical analyses; and P.E.M. conceived and interpreted all the experiments, scored pathology, and wrote and edited the manuscript.

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