In Vivo Gene Transfer Strategies to Achieve Partial Correction of von Willebrand Disease

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Abstract

von Willebrand disease (VWD), the most common hereditary coagulation disorder, results from mutations in the 52-exon gene for von Willebrand factor (VWF), which encodes an 8.4-kB cDNA. Studies with VWF cDNA plasmids have demonstrated that *in vivo* gene transfer to the liver will correct the coagulation dysfunction in VWF^{-/-} mice, but the correction is transient. To develop gene therapy for VWF that would mediate long-term expression of the VWF cDNA in liver, we first evaluated segmental pre-mRNA *trans*-splicing (SPTS) with two adeno-associated virus (AAV) serotype 8 vectors, each delivering one-half of the VWF cDNA. However, although the two vectors functioned well to generate VWF multimers after infection of cells *in vitro*, the efficiency of SPTS was insufficient to correct the VWF^{-/-} mouse *in vivo*. As an alternative, we assessed the ability of a lentiviral vector to transfer the intact murine VWF cDNA *in vivo* directly to the neonatal liver of VWF^{-/-} mice, using generation of VWF multimers, bleeding time, and bleeding volume as efficacy parameters. The VWF lentivirus generated VWF multimers and partially or completely corrected the coagulation defect on a persistent basis in 33% of the treated VWF-deficient mice. On the basis of the concept that partial persistent correction with gene transfer could be beneficial in VWD patients, these observations suggest that lentiviral delivery of VWF cDNA should be explored as a candidate for gene therapy in patients with a severe form of VWD.

Introduction

VON WILLEBRAND DISEASE (VWD) is an inherited, monogenic bleeding disorder associated with early bruising, bleeding from the nose and gums, heavy menstrual periods in women, and uncommonly, severe bleeding into joints and other internal organs (Federici, 2004; Mannucci, 2004; Sadler, 2006; Mikhail and Kouides, 2010). Although generally less severe than the classic factor VIII or IX hemophilias, the prevalence of VWD is about 1 in 100, making it the most common human hereditary coagulation disorder (Rodeghiero *et al.*, 1987; Werner *et al.*, 1993). VWD is caused by mutations in the von Willebrand factor (VWF) gene, a 52-exon, 178-kb gene located at chromosome 12p13.2 and encoding a 2050-amino acid, single-chain 250-kDa glycosylated protein (Ginsburg *et al.*, 1985; Mancuso *et al.*, 1989; Kuwano *et al.*, 1996). The VWF gene is normally expressed in megakaryocytes and endothelium (Jaffe *et al.*, 1974; Nachman *et al.*, 1977). VWF protein monomers contain O- and N-linked

glycosylation sites, and are arranged into dimers in the endoplasmic reticulum and into multimers in the Golgi apparatus via cysteine disulfide bonds of the VWF propeptides (Sadler, 1998). These multimers are stored in granules or secreted into the circulation, where they bind to coagulation factor VIII and function in hemostasis by interacting with collagen and mediating platelet adhesion at wound sites (Sakariassen *et al.*, 1979; Wise *et al.*, 1991; Sadler, 1998). Depending on the mutations in the VWF gene and the presentation of the disease, VWD is categorized into three types, with some inherited as autosomal dominant and others as autosomal recessive (Sadler and Gralnick, 1994; Sadler, 2006; Sadler *et al.*, 2006). For most affected individuals, chronic therapy is not required, but all are at risk for bleeding (Federici, 2004; Mannucci, 2004; Sadler, 2006). When needed, therapy includes partially purified VWF concentrates or desmopressin, which increases VWF plasma levels by inducing the release of VWF stored in the endothelium (Kaufmann *et al.*, 2000; Federici, 2004, 2008; Mannucci, 2004).

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Like other mendelian genetic clotting disorders, reduction of risk of VWD for bleeding could potentially be treated by gene transfer, whereby the wild-type VWF coding sequence could be transferred to affected individuals, providing persistent expression of the VWF protein to correct the systemic deficiency caused by mutations in the VWF gene (Vanden-Driessche *et al.*, 2003; Chuah *et al.*, 2011). Like the treatment of other hemophilias by gene therapy, even partial persistent correction by gene transfer could be beneficial in that it would provide a persistent increase in the baseline VWF levels, lowering the risk for bleeding and requiring less exogenous VWF to maintain normal clotting (Menache, 1998; Kay and High, 1999; Ahnstrom *et al.*, 2004; Nichols *et al.*, 2009; Nathwani *et al.*, 2011). The major challenge in developing a gene transfer strategy for VWD is that, at 8.4 kb, the size of the VWF cDNA limits the choice of vectors for *in vivo* gene transfer. On the basis of our studies (Pergolizzi *et al.*, 2006) and those of others (De Meyer *et al.*, 2008; Marx *et al.*, 2008), we know it is possible to correct the murine knockout model of VWD by *in vivo* administration of a plasmid encoding VWF administered to the liver. These studies proved it was possible to genetically modify the liver to make functional VWF capable of correcting the coagulation defects in the VWF^{-/-} mouse, but like most other *in vivo* plasmid-based gene therapy strategies, the correction was transient, lasting only days.

With this background, and the goal of providing at least partial, persistent correction of VWD, we have approached the challenge of gene therapy for VWD using two strategies, each designed to circumvent the challenge of *in vivo* gene transfer of the large VWF cDNA. First, with the knowledge that hepatocytes genetically modified with the VWF cDNA are capable of producing functional VWF (Pergolizzi *et al.*, 2006; De Meyer *et al.*, 2008; Marx *et al.*, 2008), and that adeno-associated virus type 8 (AAV8) vectors are highly efficient at transferring and expressing genes in the liver (Gao *et al.*, 2002; Sarkar *et al.*, 2004; Thomas *et al.*, 2004; Nathwani *et al.*, 2006, 2011), we devised a strategy to use AAV8 vectors to transfer the murine VWF cDNA to the liver *in vivo*. To circumvent the size limitation of AAV vectors, we used segmental pre-mRNA *trans*-splicing, with the VWF cDNA split into 5' and 3' segments (Chao *et al.*, 2002; Pergolizzi *et al.*, 2003; Pergolizzi and Crystal, 2004; Dong *et al.*, 2010; Lai *et al.*, 2010; Wu *et al.*, 2010). Assuming that the 5' and 3' vectors infect the same cell, the two expression cassettes are designed to express precursor mRNAs that *trans*-splice at the pre-mRNA level to form an intact murine VWF mRNA. Second, based on the knowledge that the intact VWF coding region could fit into a lentiviral expression cassette, we assessed the ability of a lentiviral vector to transfer the intact murine VWF transgene *in vivo* directly to the liver (Kumar *et al.*, 2001). In both strategies, we used the VWF knockout mouse as the model, with the generation of VWF multimers, bleeding time, and bleeding volume as the phenotype efficacy parameters (Denis *et al.*, 1998).

Materials and Methods

Segmental pre-mRNA *trans*-splicing 5' and 3' expression cassettes

All the plasmids were prepared with mini, maxi, or mega kits (Qiagen, Valencia, CA) according to the manufacturer's

instructions. The coding sequence of murine VWF was amplified by reverse transcription-PCR from the full-length C57BL/6 mouse VWF mRNA. The 5' and 3' segmental pre-mRNA *trans*-splicing (SPTS) constructs were designed so that the resulting two pre-mRNAs would hybridize and the nuclear spliceosome apparatus would *trans*-splice the resulting hybridized pre-mRNAs into mature, full-length murine VWF mRNA (Song *et al.*, 2009). A total of seven expression cassettes were constructed, including two for the 5' SPTS fragment and four for the 3' SPTS fragment. The engineered segmental pre-mRNA *trans*-splicing junction was located inside exon 28 (between nucleotides 4293 and 4294).

Both of the 5' SPTS expression cassettes contained a cytomegalovirus (CMV) promoter/enhancer, a 5' fragment of murine VWF cDNA (exon 1 through the 5' part of exon 28), an intron from intron 6 of human vascular endothelial growth factor gene, a foreign 50-bp hybridization binding domain (BD), and a polyadenylation signal [poly(A); Fig. 1]. The hybridization BD, a sequence described by Tahara and colleagues (2004), was used to ensure efficient splicing with a complementary hybridization BD in the 3' SPTS expression cassettes. The 5'A and 5'B constructs were similar, but to limit the size of the expression cassette, the 5'B construct was designed with the intron shortened to 27 bp from 517 bp in the 5'A construct together with a shorter rabbit β -globin poly(A) segment (SPA, 49 bp) (Levitt *et al.*, 1989; Tahara *et al.*, 2004), compared with the 224-bp bovine growth hormone (BGH) poly(A) segment in the 5'A construct.

All four of the 3' SPTS expression cassettes included a CMV promoter/enhancer, a 50-bp hybridization binding domain complementary to the one in the 5' cassette, an optimized branch point, a polypyrimidine tract, a 3' fragment of murine VWF cDNA (3' part of exon 28–exon 52), and a poly(A) sequence. The differences among the 3' SPTS constructs were the poly(A) segments and a minor difference in the length of the CMV promoter. All three 3' expression cassettes were cloned into AAV2 backbone plasmids (Kaludov *et al.*, 2002). The 3'B construct contained a 49-bp poly(A) segment based on the rabbit β -globin gene; the 3'C construct contained a 241-bp simian virus 40 (SV40) poly(A); and the 3'A and 3'D construct contained the 224-bp BGH poly(A) segment (from pcDNA3.1; Invitrogen, Carlsbad, CA).

AAV8-based SPTS 5' and 3' vectors

Preliminary *in vitro* studies of murine VWF segmental pre-mRNA *trans*-splicing in AAV plasmid-transfected cells demonstrated that cells transfected with the 5'B + 3'C AAV plasmids yielded higher VWF levels compared with the 5'B + 3'B or 5'B + 3'D pair. On the basis of these observations, the 5'B and 3'C AAV plasmids were selected to generate the AAV8-based SPTS 5' and 3' vectors AAV8-VWF 5' and AAV8-VWF 3'. The vectors were produced by a three-plasmid strategy as previously described (De *et al.*, 2006), including (1) 5'B or 3'C expression AAV plasmids; (2) pAAV8.2, a packaging plasmid that provides the AAV Rep proteins derived from AAV2 needed for vector replication as well as the viral structural (Cap) proteins VP1, VP2, and VP3 derived from AAV8 (the Cap proteins determine the serotype of the AAV vector); and (3) pAd Δ F6, an adenoviral helper plasmid that provides adenoviral helper functions of E2, E4, and V_A RNAs. To produce the AAV8-VWF SPTS

FIG. 1. 5' and 3' construction expression cassettes for segmental pre-mRNA *trans*-splicing. Shown are two 5' constructs (A and B) and four constructs (A to D). 5'A: The expression cassette (5.67 kb) includes (5' to 3'): 587-bp cytomegalovirus (CMV) promoter/enhancer, 4293-bp 5' fragment of the murine von Willebrand factor (VWF) cDNA (exon 1 to 5' of exon 28), 517-bp intron from human vascular endothelial growth factor (hVEGF) intron 6, 50-bp hybridization binding domain (BD), and 224-bp bovine growth hormone (BGH) polyadenylation signal [poly(A)]; 5'B: 5.18-kb, 680-bp CMV promoter/enhancer, 4293-bp 5' fragment of murine VWF cDNA, shortened 27-bp hVEGF intron 6, 50-bp hybridization BD and shortened 49-bp poly(A) (SPA) from rabbit β -globin gene; 3'A: 5.07-kb, 587-bp CMV promoter/enhancer, 50-bp hybridization BD that complements the BD of the 5' cassette, optimized branch point (BP), polypyrimidine tract (PPT), 4149-bp 3' fragment of murine VWF cDNA (3' of exon 28 to exon 52), and 224-bp BGH poly(A); 3'B: 5.06-kb, 680-bp CMV promoter/enhancer, 50-bp hybridization BD that complements the BD of the 5' cassette, BP, PPT, 4149-bp 3' fragment of murine VWF cDNA, and a shortened 49-bp poly(A) (SPA); 3'C: 5.16-kb, 595-bp CMV promoter/enhancer, 50-bp hybridization BD that complements the one of 5' cassette, BP, PPT, 4149-bp 3' fragment of murine VWF cDNA, and 241-bp simian virus 40 (SV40) poly(A); 3'D: 5.1-kb, 620-bp CMV promoter/enhancer, 50-bp hybridization BD that complements the BD of the 5' cassette BP, PPT, 4149-bp 3' fragment of murine VWF cDNA, and 224-bp BGH poly(A); pVWF WT, the assay positive control plasmid, is 9.25-kb, 587-bp CMV promoter/enhancer, 8442-bp full-length murine VWF cDNA, and 224-bp BGH poly(A). All constructs were in the pcDNA3.1(+) backbone and then subcloned into AAV2 packaging plasmid (Kaludov *et al.*, 2002). Color images available online at www.liebertpub.com/hum

vectors, the 5'B or 3'C expression plasmid (600 μ g), pAAV8.2 (600 μ g), and pAd Δ F6 (1.2 mg) were cotransfected with PolyFect (Qiagen) into HEK-293orf6 cells (American Type Culture Collection [ATCC], Manassas, VA); these cells contain the adenoviral E1 and E4 regions required for optimal adenoviral helper function (De *et al.*, 2006). Seventy-two hours posttransfection, the cells were harvested, and a crude viral lysate was prepared by four freeze-thaw cycles and clarified by centrifugation. The AAV8-VWF 5' and 3' vectors were purified by iodixanol gradient and QHP anion-exchange chromatography (De *et al.*, 2006). The purified vectors were concentrated with an Amicon Ultra-15 100K centrifugal filter device (Millipore, Billerica, MA) and stored in phosphate-buffered saline (PBS, pH 7.4) at -80°C . The vector genome titers were determined by quantitative TaqMan real-time PCR analysis, using a CMV promoter-specific primer-probe set (Applied Biosystems, Foster City, CA) (De *et al.*, 2006).

Lentiviral vectors

Three different transgenes were subcloned into self-inactivating lentiviral (SIN18) backbone plasmids (Naldini and Verma, 2000; Follenzi and Naldini, 2002) in order to create the lentiviral vectors. Because there are differences in the platelet receptor-binding sites between human and murine VWF, the murine VWF cDNA was used for all the experiments. The full-length 8.4-kb murine VWF cDNA, derived from the C57BL/6 mouse, was subcloned into lentiviral plasmids under the control of either the CMV or phosphoglycerate kinase (PGK) promoter/enhancer. Included 3' of the VWF gene was the woodchuck hepatitis posttranscriptional regulatory element (WPRE). The U3 region was extensively deleted, but the polyadenylation sequence was retained in the 3' long terminal repeat (LTR). The mouse VWF cDNA was cloned from the C57BL/6 mouse mRNA and the sequence was confirmed to be the wild-type murine VWF coding sequence, identical to that reported by Chitta and colleagues (2007) (GenBank AY208897).

To further assess the promoters for the lentiviral vectors *in vivo*, the human α_1 -antitrypsin (hAAT) reporter gene was used to help assess expression in C57BL/6 mice. The hAAT cDNA, encoding a secreted protein not recognized as foreign to C57BL/6 mice, was subcloned downstream of both the CMV and PGK promoters (LentiCMV.hAAT and LentiPGK.hAAT). All transgenes and their plasmids were sequenced and found to be without mutations or polymorphisms.

Production of replication-defective lentivirus was accomplished by a four-plasmid (transgene plasmid, pMDL-RRE, pRSV-Rev, and pVSV-G, at a 4:2:1:1 ratio) cotransfection of the HEK-293T cell line (ATCC), using calcium phosphate precipitation (Follenzi and Naldini, 2002). Lentivirus was harvested after 72 hr by collecting the cultured medium and concentrating it by centrifugation at 20,000 rpm for 2 hr at 4°C . Viral particle pellets were resuspended in Hanks' balanced salt solution and frozen at -80°C . All lentiviral preparations were quantified by p24 antigen levels (HIV-p24 ELISA kit; PerkinElmer, Waltham, MA), and titered by means of limiting dilution infections in additional 293T cells, and TaqMan PCR was performed with viral *gag* gene primers. Titer determinants were performed by real-time PCR (TaqMan) against the NIH3T3 cell line with one integrated

lentiviral genome (a gift from M. Sadelain, Sloan-Kettering Institute, New York, NY). Infectious titers ranged from 5×10^7 to 8×10^8 transducing units/ml.

Evaluation of vector function

The ability of the plasmid constructs to transfer the murine VWF cDNA *in vitro* was tested by assessing the ability of the vector to direct the cells to produce VWF multimers *in vitro*. HEK-293 cells (6×10^6 cells) were transfected with plasmids, using PolyFect (Qiagen). To avoid possible nonspecific binding of virus to fetal bovine serum proteins in the medium, the cells were washed once with PBS before addition of transfection medium and serum-reduced culture medium (Opti-MEM; Invitrogen) was used. Sixty to 96 hr later, the transfected cell supernatants were harvested and concentrated by passage through Amicon Ultra-4 100K centrifugal filter (Millipore) for analysis of VWF multimers.

To assess VWF multimers, agarose ([SeaKem HGT(P)], VWF grade; FMC Bioproducts, Rockland, ME) was dissolved in 40 mM Tris-acetate (pH 8.3), 0.1% sodium dodecyl sulfate, and 1 mM ethylenediaminetetraacetate (EDTA), poured to form a 4-mm-thick 1% gel, and solidified at 4°C. Samples were mixed at a 9:1 ratio with 50 mM NaH_2PO_4 (pH 7.0), 185 mM iodoacetamide, and 5.0% sodium dodecyl sulfate and were incubated at 37°C for 60 min. After incubation, 0.1 vol of 50% glycerol and 1% bromophenol blue were added to the samples. Electrophoresis was carried out for 30 min at 30 mA followed by 5 hr at 50 mA at 4°C. To transfer the proteins, 0.45- μm polyvinylidene difluoride (PVDF) transfer membrane (Amersham Hybond-P; GE Healthcare, Piscataway, NJ) was presoaked for 2 min in methanol and rinsed with transfer buffer in 2.5 mM Tris (pH 8.8), 19.2 mM glycine, 20% methanol, and 0.01% sodium dodecyl sulfate. Transfer was carried out in a Trans-Blot cell (Bio-Rad, Hercules, CA) in 2.5 mM Tris (pH 8.8), 19.2 mM glycine, 20% methanol, and 0.01% sodium dodecyl sulfate at 75 mA at 4°C overnight. After transfer, the membrane was blocked for 1 hr in 5% nonfat dry milk (Bio-Rad) diluted in 1 $\times$ PBS. Detection was accomplished by incubation, for 2 hr at 23°C with mild shaking, with a primary rabbit anti-human VWF antibody (Dako, Carpinteria, CA) diluted 1:1000 in 5% milk. After washing with 0.05% Tween-20 in 1 $\times$ PBS, the membrane was incubated, for 1 hr at 23°C with mild shaking, with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000 in 5% milk, and was washed several times with 0.05% Tween-20 in PBS. VWF multimers were visualized with enhanced chemiluminescence (ECL) Western analysis reagents (GE Healthcare) and X-ray film. Densitometric analysis of the VWF multimers in the Western analyses was carried out by scanning the X-ray film with a flat-bed scanner at 300 dpi, and the resulting .tif file was analyzed with Quantity One software (version 4.2.0; Bio-Rad), using Vertical Trace to generate a vertical cross-section of the multimers in each lane.

To assess the function of the AAV constructs *in vitro*, HEK-293orf cells (8×10^5 cells) were seeded in 6-well plates and infected with AAV8-VWF 5', AAV8-VWF 3', or AAV8-VWF 5' plus AAV8-VWF 3' for 60 min at a dose of 2.5×10^5 genome copies (GC)/cell of each vector in serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen). After infection, cells were cultured in Opti-MEM containing 10 μM

ZnCl_2 and, after 72 hr, the infected cell supernatants were harvested and concentrated by passage through an Amicon Ultra-4 100K centrifugal filter (Millipore). The supernatants were evaluated for the expression of mVWF multimers as described previously.

The lentiviral preparations were tested for transgene expression and activity by infecting human lung A549 cells. For the transduction, 200 ng of p24 per 100-mm plate (3×10^5 cells) was used to infect the cells in serum-free medium. Cultured media were harvested after 72 hr and tested for VWF expression by Western analysis. Medium was concentrated with Amicon Ultra-4 100K MWCO filters to allow for a small volume to be analyzed by gel electrophoresis.

Murine VWF model

All mice were maintained in pathogen-free environments, and the experiments were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (New York, NY). VWF knockout mice (VWF^{-/-}) on the C57BL/6 background used as a model of severe VWD were a gift from D.D. Wagner (Harvard Medical School, Boston, MA), who provided breeder pairs of mice (Denis *et al.*, 1998). These mice have a Neo^R cassette disrupting exon 4 of the murine *vwf* locus, generating progeny mice that express a completely dysfunctional VWF molecule, rendering the mice similar in phenotype to humans with severe VWD. The mice exhibit dual defects in hemostasis with highly prolonged bleeding times (>20 min) and pseudothrombophilia with markedly reduced factor VIII activity levels (FVIII:C) and activity (10 to 20% normal). Wild-type C57BL/6 mice were used as positive controls.

Evaluation of AAV8-mediated segmental pre-mRNA trans-splicing VWF expression *in vivo*

To assess whether the AAV8 vectors could mediate full VWF segmental pre-mRNA *trans-splicing in vivo*, the AAV8 SPTS vectors were administered to mice via a single tail vein injection. Experiments were performed with male or female VWF^{-/-} mice ($n=5$ per group). Mice were warmed under a heat lamp and then anesthetized with ketamine plus xylazine (100 and 10 mg/kg, respectively) by the intraperitoneal route. AAV8-VWF 5', AAV8-VWF 3', premixed AAV8-VWF 5' plus AAV8-VWF 3' at a dose of 2×10^{11} GC each, or PBS diluted in 100 μl of PBS was injected into the tail vein through an insulin syringe with a 30-gauge needle over a 30-sec period. At 2, 4, and 8 weeks postinjection, blood was collected by tail vein sampling into polypropylene tubes containing 0.1 vol of 3.8% sodium citrate. Plasma was prepared by centrifugation of the blood at $2000 \times g$ for 10 min, at 23°C. Plasma was diluted 1:2.5 in dilution buffer (2.5 mM Tris-HCl [pH 8.0] with 50 mM sodium chloride) for the assay of mVWF multimers as described previously.

Evaluation of VWF lentiviral gene expression *in vivo*

To assess newborn lentiviral intrahepatic administrations, a positive control vector containing the human AAT transgene driven by either the PGK or CMV promoter was administered to neonatal livers. Plasma levels of the AAT transgene product were monitored with an AAT ELISA (ALPCO Diagnostics, Salem, NH) (De *et al.*, 2006). Mouse plasma was diluted 1:50 in 1 $\times$ sample buffer (from the kit),

assayed in anti-AAT antibody-coated microtiter wells, and incubated for 90 min at 25°C. After washing, the captured antigens were detected with an enzyme-linked secondary antibody. Color development was terminated after 15 min in the dark and assessed at 490 nm by a microtiter plate reader (Bio-Rad). The resulting absorbance was measured against a standard of purified AAT (Brantly *et al.*, 1991) and reported as nanograms of human AAT per milliliter of plasma.

VWF lentiviral vector was administered directly into the liver of newborn VWF^{-/-} mice (1 to 2 days old), using 32-gauge needles (1 or 4 μ g of p24, 10⁹ particle units [PU]), along with recombinant human VWF protein to prevent internal bleeding (Sferra *et al.*, 2004; Wang *et al.*, 2005). The majority of the injected pups survived without any bleeding or complications and developed into adult mice. The mice were bled at 3, 8, and 12 weeks for VWF antigen detection by Western analysis, and were tested for changes in bleeding time at week 3 or week 12.

For immunohistochemical localization of VWF expression, livers were harvested 3 weeks after LentiPGK.mVWF administration, fixed overnight in 4% paraformaldehyde in PBS (EM Sciences, Hatfield, PA), and paraffin embedded, and 5- μ m sections were cut onto ProbeOn slides (Fisher Scientific, Pittsburgh, PA). For staining, the sections were cleaned and rehydrated through graded alcohol. The slides were heated in a steamer for 20 min, immersed in citrate buffer (pH 6.0), and then allowed to sit at 23°C for 20 min. Endogenous peroxidases were quenched with 0.3% H₂O₂ in PBS for 30 min at 23°C. Sections were blocked for 20 min in 5% normal goat serum in PBS. The slides were incubated overnight at 4°C in a humid chamber with rabbit anti-human VWF (Dako). The next day the slides were developed with a Vector ELITE rabbit ABC kit (Vector Laboratories, Burlingame, CA), stained with peroxidase chromogenic substrate AEC, and counterstained with Mayer's hematoxylin.

VWF bleeding time and volume

To monitor phenotypic changes in the knockout mice after gene transfer, whole blood clotting time was determined to measure the time until cessation of bleeding and total blood loss (Ware *et al.*, 2000). Mouse tails were cut ~1 cm from the terminal tip and the remainder of the tail was immersed in warmed PBS (heated to 37°C in water bath). The bleeding time was recorded as the time it took for complete cessation of blood flow. Mice that did not stop bleeding by 8 min were cauterized to prevent further blood loss. Immediately after the timed assay, 1 ml of sodium citrate was added to the saline-blood tubes to prevent clotting. The tubes were centrifuged at 2000 rpm for 10 min, and the saline layer was completely aspirated. The amount of packed red blood cells was measured manually with P200 pipets and recorded as "total RBC loss." Control mice included two wild-type (C57BL/6) and two VWF knockout mice for each bleeding time assay. Wild-type mice routinely stopped bleeding between 1 and 3 min; VWF knockout mice bled with heavy blood loss to 8 min, when the bleeding was stopped by cauterization.

Statistical analyses

All data are expressed as means $\pm$ SEM (standard error of the mean). In the bleeding time and blood loss assays, comparisons between the treated groups and controls were conducted by nonparametric one-way analysis of variance (ANOVA) using the Kruskal-Wallis test and Mann-Whitney *U* test (from <http://udel.edu/~mcdonald/statkruskalwallis.html>) because the data were not normally distributed. For comparisons of the three groups (treated, wild type, and knockout [KO]), the Kruskal-Wallis test was used with mean score equal to the mean rank of each group, and the test statistic (*H*) presenting an approximately chi-square

FIG. 2. Evaluation of the ability of combinations of the 5' and 3' VWF segmental pre-mRNA *trans*-splicing constructs to generate VWF multimers *in vitro*. **(A and B)** HEK-293 cells were transfected with plasmids and the cell media were subjected to Western analysis for VWF multimers, using rabbit anti-human VWF followed by goat anti-rabbit IgG-HRP antibodies. **(A)** Multimers assessed from cells transfected with combinations of the 5' and 3' SPTS (segmental pre-mRNA *trans*-splicing) plasmids at 96 hr posttransfection. Lane 1, mock; lane 2, transfected with 5'B; lane 3, 3'B; lane 4, 5'A; lane 5, 3'A; lane 6, 5'B + 3'B; lane 7, 5'A + 3'B; lane 8, 5'B + 3'D; lane 9, 5'A + 3'A; lane 10, positive control, full-length wild-type VWF cDNA. **(B)** Multimers from cells transfected with plasmids assayed at 60 hr posttransfection. Lane 11, mock; lane 12, 5'B; lane 13, 3'B; lane 14, 3'C; lane 15, 3'D; lane 16, 5'B + 3'B; lane 17, 5'B + 3'C; lane 18, 5'B + 3'D; lane 19, 5'A + 3'D; lane 20, positive control, full-length wild-type VWF cDNA. **(C)** HEK-293orf6 cells were infected with AAV8, and at 72 hr postinfection medium was assessed by Western analysis as described previously. Lane 21, mock; lane 22, AAV8-VWF 5'; lane 23, AAV8-VWF 3'; lane 24, both AAV8-VWF 5' and AAV8-VWF 3'; lane 25, positive control, full-length wild-type VWF cDNA.

FIG. 3. Assessment of VWF multimers generated by segmental pre-mRNA *trans*-splicing *in vivo* after intravenous administration of AAV8-VWF segmental pre-mRNA *trans*-splicing (SPTS) vectors to VWF knockout mice. AAV8-VWF 5' and/or AAV8-VWF 3' (each 2×10^{11} genome copies) or PBS was administered intravenously to VWF^{-/-} mice. At 2, 4, and 8 weeks postinjection, blood was collected and evaluated for VWF multimers by Western analysis. **(A)** Two weeks: lanes 1 and 2, naive; lane 3, PBS; lane 4, AAV8-VWF 5'; lane 5, AAV8-VWF 3'; lanes 6–10, AAV8-VWF 5' + AAV8-VWF 3'; and lane 11, plasma from wild-type (C57BL/6) mouse, positive control. **(B)** Four weeks: lane 12, naive; lane 13, PBS; lane 14, AAV8-VWF 5'; lane 15, AAV8-VWF 3'; lanes 16–20, AAV8-VWF 5' + AAV8-VWF 3'; lanes 21 and 22, plasma from wild-type (C57BL/6) mouse, positive control. **(C)** Eight weeks: lane 23, naive; lane 24, PBS; lane 25, AAV8-VWF 5'; lane 26, AAV8-VWF 3'; lanes 27–31, both AAV8-VWF 5' and AAV8-VWF 3'; and lanes 32 and 33, plasma from wild-type (C57BL/6) mouse, positive control.

FIG. 4. Lentivirus-directed expression of the murine VWF cDNA *in vitro*. **(A)** Structure of the lentiviral gene transfer vector encoding the murine VWF cDNA under the control of either the PGK (LentiPGK.mVWF) or CMV (LentiCMV.mVWF) promoter. To compare the CMV and PGK promoters *in vivo*, parallel vectors were constructed expressing the human α_1 -antitrypsin (hAAT) cDNA. The vectors (Lenti-mVWF) were generated from the SIN18 construct (Naldini and Verma, 2000; Follenzi and Naldini, 2002). The 5' LTR of the vector is chimeric, with the promoter-enhancer region of the CMV promoter replacing the U3 region (R). Upstream of the expression cassette are the splice donor site (SD), the packaging sequence (Ψ), the 5' portion of the *gag* gene with truncated reading frame including extended packaging signal (GA), the Rev response element (RRE), splice acceptor (SA), and central polypurine tract (cPPT), followed by the expression cassette containing the promoter (PGK or CMV), the full-length murine VWF cDNA or human AAT cDNA, and the posttranslational regulatory element from the woodchuck hepatitis virus (WPRE) followed by the 3' LTR with an almost complete deletion of the U3 region (SIN-18). **(B)** Expression of murine VWF from LentiPGK.mVWF and LentiCMV.mVWF *in vitro*. A549 cells (5×10^5) were infected with LentiPGK.mVWF (100 ng of p24) or LentiCMV.mVWF (100 ng of p24). After 4 days, conditioned medium was removed and 1 ml of fresh serum-free medium was added. The next day, 10 μ l of conditioned medium was analyzed on a 1% agarose-SDS gel. VWF multimers were detected by Western analysis with an anti-VWF antibody. Shown are VWF multimers in conditioned medium from lane 1, mock-infected cells; lane 2, LentiPGK.mVWF-infected cells; and lane 3, LentiCMV.mVWF-infected cells. **(C)** Comparison of serum human α_1 -antitrypsin (hAAT; α_1 AT) levels after intrahepatic administration to newborn mice of lentiviral vector with either the CMV or PGK promoter. Neonatal (day 2; $n = 6$) C57BL/6 mice were administered AAT lentiviral vector (LentiCMV.hAAT or LentiPGK.hAAT, 0.8 μ g for both) by the intrahepatic route. Serum was tested for human AAT levels. Results shown represent means $\pm$ SEM for each treated mouse group. Serum was tested for human α_1 AT levels.

distribution. For comparisons of two groups (treated vs. either wild type or KO), the Mann-Whitney *U* test was applied, which is the same as the Kruskal-Wallis test but for two variables, with the test statistic being *U* instead of *H*. Because there were many "ties" (same time or blood volume), the *H* and *U* scores were "adjusted" for the ties.

Results

To determine whether gene therapy could be used to treat von Willebrand disease *in vivo*, we developed two viral delivery systems that could accommodate the large VWF cDNA. The first strategy used adeno-associated viral (AAV) vectors with the murine VWF cDNA split at exon 28 into two parts, with splice donor, hybridization, and splice acceptor domains designed to mediate segmental pre-mRNA trans-splicing of the 5' and 3' VWF pre-mRNAs to hybridize and then splice to form a full-length VWF mRNA (Pergolizzi *et al.*, 2003; Pergolizzi and Crystal, 2004; Song *et al.*, 2009). The second strategy used lentiviruses carrying the full-length murine VWF cDNA. Both systems used murine VWF cDNA from the C57BL/6 mouse.

Evaluation of mVWF segmental pre-mRNA trans-splicing in AAV vector-infected cells

To test the feasibility of VWF segmental pre-mRNA trans-splicing, 293 cells were transfected with various combinations of the 5' and 3' expression cassettes (Fig. 1). As a positive

control, VWF Western analysis displayed high expression level multimers from cells transfected with the intact wild-type VWF cDNA (Fig. 2A, lane 10; Fig. 2B, lane 20; Fig. 2C, lane 25). As expected, multimers were not found in mock-transfected cells or cells transfected with any 5' or 3' cassette alone (Fig. 2A, lanes 1–5; Fig. 2B, lanes 11–15). However, multimers could be detected in cells transfected with any of the combinations of 5' and 3' VWF plasmids (Fig. 2A, lanes 6–9; Fig. 2B, lanes 16–19). Cells transfected with the 5'B + 3'D plasmids (Fig. 2A, lane 8) and 5'A + 3'A plasmids (Fig. 2A, lane 9) showed similar amounts of high molecular weight multimers. The intensity of bands demonstrated that the 5'B expression cassette had comparable expression efficiency as the 5'A construct even with the shorter intron and poly(A) element, which were designed to circumvent the packaging limit of AAV vectors. Cells transfected with the 5'B + 3'B constructs (Fig. 2A, lane 6) and 5'A + 3'B constructs (Fig. 2A, lane 7) showed fewer high molecular weight multimers and less intensity of bands, suggesting that the 49-bp shorter 3' SPA element likely decreases the efficiency of the 3' cassette compared with the 224-bp BGH poly(A) element in the 3'D construct. Cells transfected with 5'B + 3'C (Fig. 2B, lane 17) showed more high molecular weight multimers and also higher intensity of bands than cells transfected with 5'B + 3'B constructs (Fig. 2B, lane 16) and 5'B + 3'D constructs (Fig. 2B, lane 18), suggesting that the 3'C construct with the longer 241-bp poly(A) element has higher efficiency compared with the other two 3' cassettes with either the 49-bp SPA or 224-bp

FIG. 5. Immunohistochemical localization of VWF in the liver of VWF^{-/-} mice after intrahepatic administration of LentiPGK.mVWF to neonates. The lentiviral gene transfer vector expressing mVWF under the control of the PGK promoter (1 μ g of p24) was administered to 2-day-old VWF^{-/-} neonates via the intrahepatic route. After 3 weeks, the mice were killed and liver sections were stained with an anti-VWF antibody (B, D, and F). Control liver sections from neighboring slices containing lentiviral injected VWF^{-/-} mice liver sections (A, C, and E) were stained with anti-IgG as negative controls. Original magnification: (A–D) $\times 40$ (scale bar, 20 μ m); (E and F) $\times 100$ (scale bar, 6 μ m).

BGH poly(A) element. Thus, the 5'B + 3'C expression cassettes yielded the relatively highest amount and quality of VWF multimers. These constructs were used to generate the AAV8 *trans*-splicing vectors.

To assess AAV8-mediated VWF segmental pre-mRNA *trans*-splicing *in vitro*, 293orf6 cells were infected with either AAV8-VWF 5' alone, AAV8-VWF 3' alone, or AAV8-VWF 5' plus AAV8-VWF 3'. VWF protein expression was evaluated by Western analysis. Multimers were detected from cells infected with the combination of AAV8-VWF 5' + AAV8-VWF 3' (Fig. 2C, lane 24), but not in mock-infected cells or cells infected with either AAV8-VWF 5' or AAV8-VWF 3' alone (Fig. 2C, lanes 21–23).

Evaluation of mVWF segmental pre-mRNA *trans*-splicing mediated by AAV8 SPTS vectors *in vivo*

Our previous study demonstrated that mouse liver hepatocytes could express VWF production short term after high-volume injection of VWF-expressing plasmids into the tail vein

(Pergolizzi *et al.*, 2006). In a strategy to express VWF cDNA on a persistent basis, the AAV8 serotype was chosen to express VWF by segmental pre-mRNA *trans*-splicing *in vivo*, based on studies showing that AAV8 capsid has high tropism to hepatocytes and thus mediates high-efficiency gene transfer to liver in rodents and humans (Gao *et al.*, 2002; Sarkar *et al.*, 2004; Thomas *et al.*, 2004; Nathwani *et al.*, 2006, 2011). AAV8-VWF 5' and AAV8-VWF 3' vectors were premixed, each 2×10^{11} GC, and administered to VWF^{-/-} mice via the tail vein. At 2, 4, and 8 weeks postinjection the mice were monitored by Western analysis for plasma VWF multimers. However, despite the function of the vectors *in vitro* to generate VWF multimers (Fig. 2C), there was inefficient expression from the segmental *trans*-splicing to generate enough VWF to create visible multimers. Although there were some very low molecular weight bands from the combined vectors at 2 weeks (Fig. 3A, lanes 6–10), the mice receiving only AAV8-VWF 3' also gave a similar pattern (Fig. 3A, lane 5), and thus these are likely nonspecific. No visible multimers were detected from the combined vectors at either the 4- or 8-week time point (Fig. 3B and C).

FIG. 6. VWF multimers, bleeding time, and blood loss after intrahepatic administration of lentivirus expressing murine VWF. **(A)** Lentivirus-directed expression of murine VWF in VWF^{-/-} knockout mouse plasma. The LentiCMV.mVWF vector (1.4 μ g of p24) was administered to 2-day-old VWF^{-/-} neonates ($n=8$) via the intrahepatic route. After 12 weeks, VWF expression was assessed in plasma samples (3 μ l) by 1% agarose-SDS gel electrophoresis followed by Western analysis with anti-VWF antibody. Lanes 1 and 2, uninjected VWF^{-/-}; lanes 3–10, LentiVWF-administered VWF^{-/-} knockout mice; and lanes 11 and 12, wild-type C57BL/6 plasma samples (at 0.2 and 0.4 μ l per lane, respectively) as positive controls. **(B)** Additional examples of VWF multimers after hepatic lentivirus-mediated VWF expression in VWF^{-/-} mice. In these mice, an increased dose of lentivirus was administered (4 μ g of p24) to the neonates. After 12 weeks, VWF expression was assessed in plasma samples (6 μ l) by 1% agarose-SDS gel electrophoresis followed by Western analysis with anti-VWF antibody. Lanes 13 and 14, uninjected VWF^{-/-}; lanes 15–20, LentiCMV.mVWF-administered VWF^{-/-} knockout mice; and lanes 21 and 22, wild-type C57BL/6 mouse plasma samples (at 0.2 and 0.4 μ l per lane, respectively) as positive controls. **(C)** Densitometric analysis of examples of VWF multimers. The Western analysis in **(B)** was analyzed by densitometry to display the plasma VWF multimers quantitatively. After Western analysis development, the X-ray film was scanned with a flat-bed scanner at 300 dpi, and the resulting .tif file was analyzed with Quantity One software. Shown are scans for three different Lenti-VWF-treated mouse plasma samples (**B**, lanes 15, 18, and 19) along with one negative control (**B**, VWF naive knockout, lane 13) and a positive control (**B**, wild-type C57BL/6, lane 21). The y axis is in density units (mOD), with 100 being the baseline background for the analysis. Multimeric size is shown along the x axis (distance from top of scan, in mm), with the higher molecular weight multimers along the left side of the tracings, as a left shift. **(D and E)** Impact of LentiCMV.mVWF-directed VWF expression on hemostasis of VWF^{-/-} knockout mice. **(D)** Partial correction of bleeding time in LentiCMV.mVWF-treated neonatal knockout mice. The lentivirus-treated mice were assessed by time to cessation of bleeding after transection of the tail tip. Age-matched uninjected VWF^{-/-} mice and wild-type C57BL/6 mice were used as controls. Blood loss was significantly reduced in 9 of the 27 mice treated (33%, black dots). LentiCMV.mVWF-treated mouse bleeding time was 6.7 ± 0.4 min (SEM), with the subgroup of corrected mice showing a reduced bleeding time of 3.4 ± 0.4 min (Kruskal-Wallis test of all test groups, $H=99.1$, df 2, $p<0.0001$; comparison of treated mice with knockout negative controls, $U=20.1$, df 1, $p<0.0001$; Mann-Whitney test). For the subgroup of corrected mice ($n=9$): $H=93.4$, df 2, $p<0.0001$, Kruskal-Wallis test. Mann-Whitney test of the treated subgroup ($n=9$) compared with wild-type mice: $U=9.3$, df 1, $p=0.002$; compared with knockout negative controls: $U=63.5$, df 1, $p<0.0001$. Bleeding time means for the control groups: wild type, 1.59 ± 0.13 min, $n=39$; KO, 8.0 ± 0.0 min, $n=56$. **(E)** Correction of the bleeding diathesis in LentiCMV.mVWF-treated neonatal knockout mice as shown by reduction in overall blood loss. The mice shown in **(D)** were assayed for total blood loss after transection of the tail tip by measuring the RBC volume in the collected saline tubes. Age-matched uninjected VWF^{-/-} mice and wild-type C57BL/6 mice were used as controls. Blood loss was significantly reduced in 11 of the 27 mice treated, below that of any of the untreated KO controls (middle column), with 7 of the mice displaying blood reductions similar to that seen in the wild-type controls (left column). The mice showing complete bleeding cessation in **(D)** are shown by the black dots ($n=9$, 33% corrected). LentiPGK.mVWF-treated mouse blood loss was 133 ± 19 μ l (SEM). Kruskal-Wallis test of all groups: $H=80.2$, df 2, $p<0.0001$. Mann-Whitney test of treated mice compared with wild-type mice: $U=32.1$, df 1, $p<0.0001$; compared with knockout negative controls: $U=45.7$, df 1, $p=0.133$. For the subgroup of corrected mice ($n=9$), the Kruskal-Wallis test shows $H=93.4$, df 2, $p<0.0001$. Mann-Whitney test of treated subgroup to wild-type mice: $U=9.3$, df 1, $p=0.0022$; to knockout negative controls: $U=63.5$, df 1, $p<0.0001$. An additional six of the treated mice were observed to have low blood volumes similar to wild type (below dashed line; $n=11$, 41% of all treated mice), Kruskal-Wallis test shows $H=82.4$, df 2, $p<0.0001$. Mann-Whitney test of low-volume subgroup ($n=11$) to wild-type mice: $U=7.9$, df 1, $p=0.0049$; to knockout negative controls: $U=5.2$, df 1, $p=0.023$. Blood volume means for the control groups: wild type, 16 ± 1.7 μ l, $n=42$; KO, 206.0 ± 9.0 μ l, $n=55$. Color images available online at www.liebertpub.com/hum

Expression of mVWF lentivirus in vitro

PCR analysis of cells transduced *in vitro* with lentiviral DNA showed the presence of both viral genes and VWF exons (data not shown). Lentiviral titering of these vectors by limiting dilutions and by p24 antigen ELISA indicated high infectivity (10^9 PU/ml) and yields of 18–30 ng of p24 particles/ml. No apparent loss of infectivity or low yields were observed with the full-length VWF lentiviruses. Transduction of the VWF lentiviral vectors (Fig. 4A) into human lung carcinoma line A549 mediated robust expression of full-

length VWF multimers by either promoter (PGK or CMV; Fig. 4B, lanes 2 and 3).

Lentivirus-mediated gene transfer in vivo

As a comparison of the ability of the PGK versus CMV promoter VWF lentiviral vectors to function in hepatic gene transfer *in vivo*, lentiviruses expressing hAAT under the control of either of the two promoters were administered by direct hepatic administration to neonatal mice. Using AAT as a surrogate secreted plasma protein for VWF, we observed

that the expression levels with both promoters remained stable for at least 12 weeks, at about 1 μ g of hAAT per milliliter (Fig. 4C).

To assess lentivirus-mediated correction of the VWF^{-/-} mouse, newborn VWF^{-/-} mice were injected intrahepatically with lentiviruses expressing the VWF cDNA (1 or 4 μ g of p24 or 1–2 $\times 10^9$ PU), plus recombinant human VWF protein (5 μ g, to prevent internal bleeding). Almost all of the injected pups survived without any bleeding or complications and developed into adult mice. Immunohistochemistry was performed on serial liver sections from lentivirus-transduced and negative control VWF^{-/-} mice, using anti-VWF or irrelevant IgG antibodies (Fig. 5). Deposits of the antibody (brown to red color) were observed within most hepatocytes in the liver sections from Lenti-VWF-administered mice, especially in the hepatocytes around portal triads, indicative of positive antibody detection of VWF proteins (Fig. 5B and D compared with Fig. 5A and C, antibody control). The LentiPGK.mVWF-treated mouse livers also had abundant areas of positive staining in the endothelial cells lining portal veins (compare Fig. 5F with 5E). No staining was observed in nontreated control VWF^{-/-} livers (data not shown).

On the basis of the positive immunohistochemistry findings of VWF expression in liver, the neonatal injections were repeated in newborn mice and assessed over time for detectable levels of plasma VWF and phenotypic correction of bleeding times. Increasing doses of Lenti-VWF (1 to 4 μ g) were administered by direct intrahepatic injection in neonates. The Lenti-VWF-injected mice were monitored by Western analysis once every 4 weeks for plasma VWF multimers and by bleeding time assays once at 12 weeks. No significant differences were observed with the CMV and PGK promoters; only the CMV data are shown (Fig. 6).

The results were variable regardless of the dose administered. Several mice of each neonatal litter developed long-term expression of VWF multimers (e.g., see Fig. 6A, lanes 3, 8, and 9, and Fig. 6B, lanes 15, 16, 18, 19, and 20); whereas other mice failed to develop detectable VWF multimers. The highest number of positive VWF responders occurred with the use of higher lentivirus dose (4 μ g; Fig. 6B). Densitometry showed that the lower molecular weight multimers from the LentiCMV.mVWF-treated mice were distinct from the background of the VWF^{-/-} mice (see, e.g., Fig. 6C, lane 13 [negative] vs. lanes 15, 18, and 19 [treated]). In the wild-type VWF^{+/+} plasma from C57BL/6 mice, medium weight multimers were more prevalent, but the lower molecular weight multimers match up with the lower molecular weight multimers from the Lenti-VWF-treated mouse plasma (Fig. 6C, lanes 18 and 19 [treated] vs. lane 21 [positive]).

By week 12 posttreatment, 9 of 27 mice from 4 separate litters displayed complete bleeding cessation during the tail snip bleeding time assays. An additional 3 of the 27 treated mice displaying bleeding cessation that was reduced but not normalized compared with age-matched wild-type C57BL/6 control mice (Fig. 6D). When these LentiCMV.mVWF-treated neonate litters were compared for overall blood loss during the bleeding assay, 11 of the 27 treated adult mice showed reductions (RBC volume, <100 μ l) far below that observed with knockout negative VWF^{-/-} controls, with 5 of these treated mice demonstrating near wild-type mouse blood loss levels (Fig. 6E). Thus, approximately one-third of the

VWF^{-/-} mice demonstrated partial or complete reversal of the severe VWF coagulation defect.

Discussion

Because von Willebrand disease is a monogenetic disorder with a phenotype easily assessed in blood, it could be an ideal candidate for gene therapy (Federici, 2004; Mannucci, 2004; Sadler, 2006; Mikhail and Kouides, 2010). The challenge in achieving this is the large size of the VWF cDNA (~8.4 kb) which, together with an active promoter, conflicts with packing constraints in most viral gene transfer vectors (Kumar *et al.*, 2001; Dong *et al.*, 2010; Lai *et al.*, 2010; Wu *et al.*, 2010). In the present study, we examined two different approaches to viral vector delivery of the VWF cDNA to correct the coagulation-related phenotype of the VWF^{-/-} mouse. First, in order to take advantage of the efficiency of AAV8 vectors to transfer genes to hepatocytes *in vivo* (Thomas *et al.*, 2004; Nathwani *et al.*, 2006, 2011), but to circumvent the packaging constraints of AAV vectors, we split the VWF cDNA and expressed the 5' and 3' fragments in two AAV8 vectors using segmental pre-mRNA *trans*-splicing, using a hybridization domain and splice donor and acceptor site to fuse the two fragments at the pre-mRNA level. However, although effective *in vitro* in generating VWF multimers, the SPTS strategy *in vivo* was not efficient enough to generate detectable VWF multimers or to correct the coagulation defects of the VWF^{-/-} mice. As a second strategy, we expressed the full-length murine VWF cDNA in a lentiviral vector. Not only did the lentivirus-VWF vector generate VWF multimers *in vitro*, but direct hepatic administration to neonatal VWF^{-/-} mice resulted in the generation of VWF protein in hepatocytes and liver endothelium, and approximately 33% of the treated mice generated VWF multimers in serum and partial or full correction of the coagulation defect. Although improvements in efficiency will have to be made, all together the data demonstrate the potential of lentiviral delivery of VWF cDNA as a candidate for gene therapy to at least partially treat severe VWD.

Prior attempts of cell and gene therapy for von Willebrand disease

Bone marrow transplants of wild-type marrow into pigs with VWD have been attempted with limited success (Bowie *et al.*, 1986; Nichols *et al.*, 1995). Lin and colleagues (2002) established that cultured autologous "blood outgrowth endothelial cells" (BOECs) isolated from blood maintained expression of VWF through multiple passages, and De Meyer and colleagues (2006) used lentiviral transduction of the canine-VWF cDNA to correct VWF-deficient cultured canine BOECs *in vitro*. Although the *ex vivo* approach to treat VWD with genetically corrected BOECs is possible, large numbers of these cells will be needed as there is no selective advantage to the transfected cell progeny, and successful therapy will therefore require repeated infusions of large numbers of genetically modified cells to produce therapeutic VWF plasma levels.

Transient *in vivo* correction of the mouse model of VWD has been achieved by high-volume tail vein administration to deliver to the liver plasmids expressing the VWF cDNA under the control of an active promoter. Our group (Pergolizzi *et al.*, 2006), Marx and colleagues (2008), and De Meyer

and colleagues (2008) demonstrated correction of VWD in VWF-deficient mice by this strategy. However, in all of these studies, there was only transient expression of VWF and correction of the VWF^{-/-} mouse was observed for less than 2 weeks.

Gene therapy for von Willebrand disease using segmental pre-mRNA trans-splicing vectors

SPTS is a strategy to get around the insert size limitations of AAV vectors, typically 4.7 to 5.3 kb (Grieger and Samulski, 2005; Dong *et al.*, 2010; Lai *et al.*, 2010; Wu *et al.*, 2010) and significantly smaller than the cDNA for VWF (8.4 kb). In an attempt to adapt the SPTS strategy to treat VWD, we inserted 5' and 3' fragments of the murine VWF cDNA into two AAV8 vectors as a segmental pre-mRNA *trans*-splicing delivery system. This successfully produced VWF monomers *in vitro*, but failed to efficiently express detectable levels of VWF multimers in VWF knockout mice. This result is likely due to an insufficient level of cells coinfecting with both the 5' and 3' coding AAV8 vectors. In contrast, *trans*-splicing of large genes using dual AAV vectors has been achieved in muscle by using AAV9 vectors encoding the 5' and 3' segments of alkaline phosphatase (Ghosh *et al.*, 2007, 2009) and AAV6 vectors encoding dystrophin (Zhang and Duan, 2012). Whether the greater success of these studies compared with our AAV8-mediated segmental pre-mRNA *trans*-splicing to liver was due to the relative ease of detection of the transgenes used (intracellular vs. secreted for VWF), to the AAV serotype, and/or to the differences in the organs (liver vs. muscle) being treated is unclear. For segmental pre-mRNA *trans*-splicing of the VWF cDNA with AAV8, the inefficiency observed could theoretically be overcome by increasing the dose of the two vectors, but such high doses may be inappropriate for human clinical use for safety reasons. Thus, we have abandoned the SPTS AAV8 strategy for *in vivo* treatment of VWD.

In vivo gene therapy for von Willebrand disease with lentiviral vectors

A promising approach to *in vivo* gene therapy for large genes is to use replication-defective lentiviruses, with a packaging constraint of 8.5 to 9.7 kb (Kumar *et al.*, 2001). Using a third-generation lentivirus, our studies focused on the use of *in vivo* lentivirus-mediated expression of the mouse VWF transgene in the liver of neonatal VWF^{-/-} mice. Experimental trials in mice and rabbits have demonstrated that *in vivo* lentiviral gene therapy can be successful for targeting adult and neonatal livers, including *in vivo* gene transfer of marker genes such as GFP and eGFP, as well as correcting factor VIII and IX deficiency and Fabry's disease (α -galactosidase) (Kafri *et al.*, 1997; Kootstra *et al.*, 2003; Chen *et al.*, 2004; Waddington *et al.*, 2004; Yoshimitsu *et al.*, 2004; Salani *et al.*, 2005; Moreno *et al.*, 2008). Salani and colleagues (2005) demonstrated that lentivirus could mediate transgene expression in the liver of neonatal mice, and Moreno and colleagues (2008) found that lentivirus could be used *in utero* to transfer genes to the fetus. Intrahepatic administration to the neonatal liver has been successful in rabbits (Moreno *et al.*, 2008). Relevant to treatment of VWD, Niiya and colleagues (2009) used lentiviruses to deliver the VWF-cleaving enzyme ADAMTS13 by the intravenous route *in utero* to

fetal ADAMTS13-deficient mice, which led to liver transduction by the lentiviral vector with generation of normal VWF multimeric status.

We directly administered lentivirus into neonatal liver to deliver the lentiviral mVWF transgene to hepatocytes and liver sinusoidal endothelium. Direct administration of the lentiviral-VWF into 2-day neonatal liver demonstrated production of VWF in liver hepatocytes and endothelial cells, with consequent partial or complete correction of the coagulation defect in approximately one-third of the mice. The variability of the response in the inbred VWF^{-/-} mice to therapy with the lentiviral vector is an interesting observation, but it has been observed in other studies (Kootstra *et al.*, 2003; Waddington *et al.*, 2004). Given that only 5% of the wild-type VWF level is necessary to correct the defect, it is likely that what we are observing is a critical threshold of correction, where the two-thirds of treated VWF^{-/-} mice are not expressing adequate VWF protein levels to observe a corrected phenotype. Thus, a higher "cure rate" may be achievable by increasing the dose, modifying elements in the vectors, and/or repeat dosing.

VWD is an inherited disorder associated with a lifelong predisposition to mucocutaneous bleeds (Federici, 2004; Mannucci, 2004; Sadler, 2006; Mikhail and Kouides, 2010). Depending on the severity of the disease, the patient may require daily to weekly administration of VWF concentrates or plasma cryoprecipitates, with the associated prohibitive costs (Mannucci, 2004; Mirchandani *et al.*, 2011). Even small improvements in local production of VWF could alleviate at least some of the consequences of the coagulation defect, suggesting it is worthwhile to continue to evaluate *in vivo* lentivirus VWF gene transfer as a possible therapy for severe VWD.

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Author Disclosure Statement

The authors have no conflicts to disclose.

References

- Ahnstrom, J., Berntorp, E., Lindvall, K., *et al.* (2004). A 6-year follow-up of dosing, coagulation factor levels and bleedings in relation to joint status in the prophylactic treatment of haemophilia. *Haemophilia* 10, 689–697.
- Bowie, E.J., Solberg, L.A., Jr., Fass, D.N., *et al.* (1986). Transplantation of normal bone marrow into a pig with severe von Willebrand's disease. *J. Clin. Invest.* 78, 26–30.
- Brantly, M.L., Wittes, J.T., Vogelmeier, C.F., *et al.* (1991). Use of a highly purified α_1 -antitrypsin standard to establish ranges for the common normal and deficient α_1 -antitrypsin phenotypes. *Chest* 100, 703–708.
- Chao, H., Sun, L., Bruce, A., *et al.* (2002). Expression of human factor VIII by splicing between dimerized AAV vectors. *Mol. Ther.* 5, 716–722.

- Chen, X.G., Zhu, H.Z., Gong, J.L., *et al.* (2004). Efficient delivery of human clotting factor IX after injection of lentiviral vectors *in utero*. *Acta Pharmacol. Sin.* 25, 789–793.
- Chitta, M.S., Duhe, R.J., and Kermode, J.C. (2007). Cloning of the cDNA for murine von Willebrand factor and identification of orthologous genes reveals the extent of conservation among diverse species. *Platelets* 18, 182–198.
- Chuah, M.K.L., Petrus, I., and Vandendriessche, T. (2011). Gene therapy for von Willebrand disease. In *von Willebrand Disease: Basic and Clinical Aspects*, A.B. Federici, C.A. Lee, E.E. Bernthorpe, D. Lillicrap, and R.R. Montgomery, eds. (Wiley-Blackwell, Oxford, UK), Ch. 20.
- De, B.P., Heguy, A., Hackett, N.R., *et al.* (2006). High levels of persistent expression of α_1 -antitrypsin mediated by the non-human primate serotype rh.10 adeno-associated virus despite preexisting immunity to common human adeno-associated viruses. *Mol. Ther.* 13, 67–76.
- De Meyer, S.F., Vanhoorelbeke, K., Chuah, M.K., *et al.* (2006). Phenotypic correction of von Willebrand disease type 3 blood-derived endothelial cells with lentiviral vectors expressing von Willebrand factor. *Blood* 107, 4728–4736.
- De Meyer, S.F., Vandeputte, N., Pareyn, I., *et al.* (2008). Restoration of plasma von Willebrand factor deficiency is sufficient to correct thrombus formation after gene therapy for severe von Willebrand disease. *Arterioscler. Thromb. Vasc. Biol.* 28, 1621–1626.
- Denis, C., Methia, N., Frenette, P.S., *et al.* (1998). A mouse model of severe von Willebrand disease: Defects in hemostasis and thrombosis. *Proc Natl. Acad. Sci. U.S.A.* 95, 9524–9529.
- Dong, B., Nakai, H., and Xiao, W. (2010). Characterization of genome integrity for oversized recombinant AAV vector. *Mol. Ther.* 18, 87–92.
- Federici, A.B. (2004). Clinical diagnosis of von Willebrand disease. *Haemophilia* 10(Suppl. 4), 169–176.
- Federici, A.B. (2008). The use of desmopressin in von Willebrand disease: The experience of the first 30 years (1977–2007). *Haemophilia* 14(Suppl. 1), 5–14.
- Follenzi, A., and Naldini, L. (2002). Generation of HIV-1 derived lentiviral vectors. *Methods Enzymol.* 346, 454–465.
- Gao, G.P., Alvira, M.R., Wang, L., *et al.* (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11854–11859.
- Ghosh, A., Yue, Y., Long, C., *et al.* (2007). Efficient whole-body transduction with *trans*-splicing adeno-associated viral vectors. *Mol. Ther.* 15, 750–755.
- Ghosh, A., Yue, Y., Shin, J.H., *et al.* (2009). Systemic *trans*-splicing adeno-associated viral delivery efficiently transduces the heart of adult *mdx* mouse, a model for Duchenne muscular dystrophy. *Hum. Gene Ther.* 20, 1319–1328.
- Ginsburg, D., Handin, R.I., Bonthron, D.T., *et al.* (1985). Human von Willebrand factor (vWF): Isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science* 228, 1401–1406.
- Grieger, J.C., and Samulski, R.J. (2005). Packaging capacity of adeno-associated virus serotypes: Impact of larger genomes on infectivity and postentry steps. *J. Virol.* 79, 9933–9944.
- Jaffe, E.A., Hoyer, L.W., and Nachman, R.L. (1974). Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 71, 1906–1909.
- Kafri, T., Blomer, U., Peterson, D.A., *et al.* (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* 17, 314–317.
- Kaludov, N., Handelsman, B., and Chiorini, J.A. (2002). Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum. Gene Ther.* 13, 1235–1243.
- Kaufmann, J.E., Oksche, A., Wollheim, C.B., *et al.* (2000). Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. *J. Clin. Invest.* 106, 107–116.
- Kay, M.A., and High, K. (1999). Gene therapy for the hemophilias. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9973–9975.
- Kootstra, N.A., Matsumura, R., and Verma, I.M. (2003). Efficient production of human FVIII in hemophilic mice using lentiviral vectors. *Mol. Ther.* 7, 623–631.
- Kumar, M., Keller, B., Makalou, N., *et al.* (2001). Systematic determination of the packaging limit of lentiviral vectors. *Hum. Gene Ther.* 12, 1893–1905.
- Kuwano, A., Morimoto, Y., Nagai, T., *et al.* (1996). Precise chromosomal locations of the genes for dentatorubral-pallidolysian atrophy (DRPLA), von Willebrand factor (F8vWF) and parathyroid hormone-like hormone (PTHrP) in human chromosome 12p by deletion mapping. *Hum. Genet.* 97, 95–98.
- Lai, Y., Yue, Y., and Duan, D. (2010). Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome ≥ 8.2 kb. *Mol. Ther.* 18, 75–79.
- Levitt, N., Briggs, D., Gil, A., *et al.* (1989). Definition of an efficient synthetic poly(A) site. *Genes Dev.* 3, 1019–1025.
- Lin, Y., Chang, L., Solovey, A., *et al.* (2002). Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. *Blood* 99, 457–462.
- Mancuso, D.J., Tuley, E.A., Westfield, L.A., *et al.* (1989). Structure of the gene for human von Willebrand factor. *J. Biol. Chem.* 264, 19514–19527.
- Mannucci, P.M. (2004). Treatment of von Willebrand's disease. *N. Engl. J. Med.* 351, 683–694.
- Marx, I., Lenting, P.J., Adler, T., *et al.* (2008). Correction of bleeding symptoms in von Willebrand factor-deficient mice by liver-expressed von Willebrand factor mutants. *Arterioscler. Thromb. Vasc. Biol.* 28, 419–424.
- Menache, D. (1998). Pharmacokinetics of von Willebrand factor and factor VIII coagulant activity in patients with von Willebrand disease type 3 and type 2. *Haemophilia* 4(Suppl. 3), 44–47.
- Mikhail, S., and Kouides, P. (2010). von Willebrand disease in the pediatric and adolescent population. *J. Pediatr. Adolesc. Gynecol.* 23, S3–S10.
- Mirchandani, G.G., Drake, J.H., Cook, S.L., *et al.* (2011). Surveillance of bleeding disorders, Texas, 2007. *Am. J. Prev. Med.* 41, S354–S359.
- Moreno, R., Rosal, M., Martinez, I., *et al.* (2008). Restricted transgene persistence after lentiviral vector-mediated fetal gene transfer in the pregnant rabbit model. *J. Gene Med.* 10, 951–964.
- Nachman, R., Levine, R., and Jaffe, E.A. (1977). Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *J. Clin. Invest.* 60, 914–921.
- Naldini, L., and Verma, I.M. (2000). Lentiviral vectors. *Adv. Virus Res.* 55, 599–609.
- Nathwani, A.C., Gray, J.T., Ng, C.Y., *et al.* (2006). Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 107, 2653–2661.
- Nathwani, A.C., Tuddenham, E.G., Rangarajan, S., *et al.* (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N. Engl. J. Med.* 365, 2357–2365.

- Nichols, T.C., Samama, C.M., Bellinger, D.A., *et al.* (1995). Function of von Willebrand factor after crossed bone marrow transplantation between normal and von Willebrand disease pigs: Effect on arterial thrombosis in chimeras. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2455–2459.
- Nichols, T.C., Dillow, A.M., Franck, H.W., *et al.* (2009). Protein replacement therapy and gene transfer in canine models of hemophilia A, hemophilia B, von Willebrand disease, and factor VII deficiency. *ILAR J.* 50, 144–167.
- Niyya, M., Endo, M., Shang, D., *et al.* (2009). Correction of ADAMTS13 deficiency by *in utero* gene transfer of lentiviral vector encoding ADAMTS13 genes. *Mol. Ther.* 17, 34–41.
- Pergolizzi, R.G., and Crystal, R.G. (2004). Genetic medicine at the RNA level: Modifications of the genetic repertoire for therapeutic purposes by pre-mRNA *trans*-splicing. *C.R. Biol.* 327, 695–709.
- Pergolizzi, R.G., Ropper, A.E., Dragos, R., *et al.* (2003). *In vivo trans*-splicing of 5' and 3' segments of pre-mRNA directed by corresponding DNA sequences delivered by gene transfer. *Mol. Ther.* 8, 999–1008.
- Pergolizzi, R.G., Jin, G., Chan, D., *et al.* (2006). Correction of a murine model of von Willebrand disease by gene transfer. *Blood* 108, 862–869.
- Rodeghiero, F., Castaman, G., and Dini, E. (1987). Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood* 69, 454–459.
- Sadler, J.E. (1998). Biochemistry and genetics of von Willebrand factor. *Annu. Rev. Biochem.* 67, 395–424.
- Sadler, J.E. (2006). von Willebrand disease. In *Scriver's Online Metabolic and Molecular Bases of Inherited Disease*, D. Valle, A.L. Beaudet, B. Vogelstein, K.W. Kinzler, *et al.*, eds. (McGraw-Hill, New York, NY).
- Sadler, J.E., and Gralnick, H.R. (1994). Commentary: A new classification for von Willebrand disease. *Blood* 84, 676–679.
- Sadler, J.E., Budde, U., Eikenboom, J.C., *et al.* (2006). Update on the pathophysiology and classification of von Willebrand disease: A report of the Subcommittee on von Willebrand Factor. *J. Thromb. Haemost.* 4, 2103–2114.
- Sakariassen, K.S., Bolhuis, P.A., and Sixma, J.J. (1979). Human blood platelet adhesion to artery subendothelium is mediated by factor VIII–von Willebrand factor bound to the sub-endothelium. *Nature* 279, 636–638.
- Salani, B., Damonte, P., Zingone, A., *et al.* (2005). Newborn liver gene transfer by an HIV-2-based lentiviral vector. *Gene Ther.* 12, 803–814.
- Sarkar, R., Tetreault, R., Gao, G., *et al.* (2004). Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* 103, 1253–1260.
- Sferra, T.J., Backstrom, K., Wang, C., *et al.* (2004). Widespread correction of lysosomal storage following intrahepatic injection of a recombinant adeno-associated virus in the adult MPS VII mouse. *Mol. Ther.* 10, 478–491.
- Song, Y., Lou, H.H., Boyer, J.L., *et al.* (2009). Functional cystic fibrosis transmembrane conductance regulator expression in cystic fibrosis airway epithelial cells by AAV6.2-mediated segmental *trans*-splicing. *Hum. Gene Ther.* 20, 267–281.
- Tahara, M., Pergolizzi, R.G., Kobayashi, H., *et al.* (2004). *Trans*-splicing repair of CD40 ligand deficiency results in naturally regulated correction of a mouse model of hyper-IgM X-linked immunodeficiency. *Nat. Med.* 10, 835–841.
- Thomas, C.E., Storm, T.A., Huang, Z., *et al.* (2004). Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J. Virol.* 78, 3110–3122.
- VandenDriessche, T., Collen, D., and Chuah, M.K. (2003). Gene therapy for the hemophilias. *J. Thromb. Haemost.* 1, 1550–1558.
- Waddington, S.N., Nivsarkar, M.S., Mistry, A.R., *et al.* (2004). Permanent phenotypic correction of hemophilia B in immunocompetent mice by prenatal gene therapy. *Blood* 104, 2714–2721.
- Wang, Z., Zhu, T., Qiao, C., *et al.* (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat. Biotechnol.* 23, 321–328.
- Ware, J., Russell, S., and Ruggeri, Z.M. (2000). Generation and rescue of a murine model of platelet dysfunction: The Bernard–Soulier syndrome. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2803–2808.
- Werner, E.J., Broxson, E.H., Tucker, E.L., *et al.* (1993). Prevalence of von Willebrand disease in children: A multiethnic study. *J. Pediatr.* 123, 893–898.
- Wise, R.J., Dorner, A.J., Krane, M., *et al.* (1991). The role of von Willebrand factor multimers and propeptide cleavage in binding and stabilization of factor VIII. *J. Biol. Chem.* 266, 21948–21955.
- Wu, Z., Yang, H., and Colosi, P. (2010). Effect of genome size on AAV vector packaging. *Mol. Ther.* 18, 80–86.
- Yoshimitsu, M., Sato, T., Tao, K., *et al.* (2004). Bioluminescent imaging of a marking transgene and correction of Fabry mice by neonatal injection of recombinant lentiviral vectors. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16909–16914.
- Zhang, Y., and Duan, D. (2012). Novel mini-dystrophin gene dual adeno-associated virus vectors restore neuronal nitric oxide synthase expression at the sarcolemma. *Hum. Gene Ther.* 23, 98–103.

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