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Phenotypic Correction of Hemophilia A in Sheep by Postnatal Intraperitoneal Transplantation of FVIII-Expressing MSC

Christopher D. Porada, PhD^{1,2}, Chad Sanada, PhD², Chung-Jung Kuo, MS^{1,2}, Evan Colletti, PhD², Walter Mandeville, DVM³, John Hasenau, DVM³, Esmail D. Zanjani, PhD², Robert Moot, BS⁴, Christopher Doering, PhD⁵, H. Trent Spencer, PhD⁶, and Graça Almeida-Porada, MD, PhD^{1,2}

¹Wake Forest Institute for Regenerative Medicine, 391 Technology Way, Winston-Salem, NC 27157-1083

²Dept. of Animal Biotechnology, University of Nevada, 1664 N. Virginia Street, MS 202, Reno, NV 89557-0104

³Dept. of Laboratory Animal Medicine, University of Nevada, 1664 N. Virginia Street, MS 340, Reno, NV 89557

⁴Dept. of Pediatrics, Aflac Cancer Center and Blood Disorders Service, 1405 Clifton Road NE, Atlanta, GA 30322

⁵Emory Children's Center Pediatrics, 2015 East Gate Court SE, Atlanta, GA 30322

⁶Dept. of Pediatrics, Emory University School of Medicine, 201 Dowman Drive. Atlanta, GA 30322

Abstract

We recently re-established a line of sheep that accurately mimics the clinical symptoms and genetics of severe hemophilia A (HA). Herein, we tested a novel, non-ablative transplant therapy in 2 pediatric HA animals. Paternal mesenchymal stem cells (MSC) were transduced with a porcine FVIII-encoding lentivector, and transplanted via the intraperitoneal route, without preconditioning. At the time of transplantation, these animals had received multiple hFVIII treatments for various spontaneous bleeds, and had developed debilitating hemarthroses which produced severe defects in posture and gait. Transplantation of transduced MSC resolved all existent hemarthroses, and spontaneous bleeds ceased. Damaged joints recovered fully; the animals regained normal posture and gait and resumed normal activity. Despite achieving factor-independence, a sharp rise in pre-existent Bethesda titers occurred following transplantation, decreasing the effectiveness and duration of therapy. Post-mortem examination revealed widespread engraftment, with MSC present within the lung, liver, intestine, and thymus, but particularly within joints affected at the time of transplantation, suggesting MSC homed to sites of ongoing injury/inflammation to release FVIII, explaining the dramatic improvement in hemarthrotic joints. In summary, this novel, non-ablative MSC transplantation was

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To whom correspondence should be addressed: Christopher D. Porada, Ph.D., Associate Professor, Wake Forest Institute for Regenerative Medicine, Richard H. Dean Biomedical Building, 4th Floor, 391 Technology Way, Winston-Salem, NC 27157-1083, cporada@wfubmc.edu.

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straightforward, safe, and converted life-threatening, debilitating HA to a moderate phenotype in a large animal model.

Keywords

Hemophilia A; large animal model; mesenchymal stem cells; gene therapy; non-ablative transplant

Introduction

Hemophilia A (HA) is the most common inheritable deficiency of coagulation [1]. Individuals with severe HA experience frequent hemorrhaging, leading to chronic debilitating arthropathy, hematomas of the subcutaneous connective tissue/muscle, and internal bleeding. While current treatments allow many patients with HA to live relatively normal lives, they are far from ideal, due to the need for lifelong infusions, the high cost, and the high incidence of FVIII inhibitors [2]. Furthermore, replacement therapy is not available for ~75% of HA patients worldwide, placing these patients at great risk for severe and permanent disabilities. There is thus an urgent need for novel HA therapies offering longer-lasting benefit or permanent cure [3-6]. Because precise transcriptional regulation and cell-specific expression of FVIII are not required, and since even modest levels of FVIII-expressing cells could convert patients with severe HA to a moderate/mild phenotype, greatly improving quality of life, HA is considered an ideal disease for correction by gene therapy [7].

Several characteristics of mesenchymal stem cells (MSC) make them well-suited as gene delivery vehicles, especially in the context of HA: 1) their ease of isolation from the bone marrow; 2) their ability to expand exponentially in vitro to adequate numbers for clinical application; 3) they produce robust, durable engraftment in multiple organs following transplantation and contribute to both the parenchyma and the perivascular zones of the engrafted organs [8-12], placing them ideally for delivering FVIII into the circulation; 4) MSC can be transduced with FVIII-encoding viral vectors and subsequently secrete high levels of biologically active FVIII [4, 13, 14]; 5) the well documented immunomodulatory properties of MSC [15-18] may make them uniquely suited for the delivery of FVIII, possibly reducing the potential for inhibitor formation or, perhaps, even allowing the use of FVIII gene delivery in the large number of HA patients harboring FVIII inhibitors; and 6) in contrast to hematopoietic cells, there is no evidence that MSC transform or progress to clonal dominance following transduction with integrating viral vectors. On the contrary, even if genomic instability is intentionally induced, MSC undergo terminal differentiation rather than transformation [19], suggesting they represent inherently safer cellular vehicles for delivering FVIII.

The ideal way to test the clinical viability of novel HA treatments is in an animal model that parallels both the clinical symptoms of HA and normal human physiology. We recently re-established a line of sheep with severe HA, and fully characterized both the clinical parameters and the molecular basis for their disease [20]. In similarity to mutations seen in many human patients [21], these sheep possess a premature stop codon with a frame shift mutation, making them unique among preclinical models for HA.

In the present studies, we used this line of HA sheep to test a novel, non-ablative transplant-based gene therapy in 2 pediatric HA animals, transplanting the HA animals with haploidentical (paternal) bone marrow-derived MSC engineered to express porcine FVIII (pFVIII). We show that this simple treatment, involving only an intraperitoneal injection with no preconditioning, resulted in cessation of spontaneous bleeds and complete reversal

of the joint stiffness/swelling and the gait/postural defects that had developed as a result of frequent hemarthroses during the first months of life. Post-mortem examination revealed high levels of engraftment within joints affected at the time of transplantation, suggesting MSC were able to home to sites of ongoing injury/inflammation and release FVIII locally. Despite achieving factor-independence, however, a sharp rise in Bethesda titers occurred following transplantation, likely decreasing the effectiveness and duration of therapy.

In summary, the non-ablative MSC transplantation we describe was straightforward and converted life-threatening, debilitating HA to a moderate phenotype in a large animal model. The simplicity/safety of this procedure and the marked phenotypic correction it mediated make the present studies of high potential clinical impact. However, further mechanistic studies will be required to understand and ultimately overcome the formation of inhibitors resultant from this procedure since, in the absence of inhibitors, this approach could result in even more pronounced and longer-lasting clinical improvement.

Materials and Methods

Isolation and Culture of Sheep BM-MSC

Bone marrow (BM) was collected from the ram that sired both sheep transplanted in these studies. BM mononuclear cells were isolated by Ficoll density gradient (Histopaque 1077, Sigma, St. Louis, MO), immunomagnetically depleted of CD45+ and GlyA+ cells (Miltenyi Biotec, Auburn, CA), and plated in gelatin-coated flasks (BD Falcon, San Jose, CA) in MSC Growth Media (MSCGM, Lonza, Walkersville, MD). Media was changed after 24 hours to remove non-adherent cells. Upon reaching 70% confluence, cells were passaged using 0.25% trypsin (Gibco/Invitrogen, Carlsbad, CA).

Transduction of MSC with high-expression porcine FVIII vector

MSC were transduced three times, during passages 1-3, with a SIN HIV-based vector encoding an expression/secretion optimized B-domain deleted porcine FVIII (pFVIII) gene [22], and once with an identical lentivector encoding eGFP, to facilitate MSC tracking after transplantation. Expression of the transgene was driven by an internal CMV promoter in each vector. FVIII production by transduced cells was quantitated using a commercially available chromogenic assay according to the manufacturer's instructions (COATEST Factor VIII, Chromogenix), and transduction efficiency was determined by Q-PCR as previously described [5] utilizing untransduced sheep MSC as a negative control to confirm the absolute specificity of the primers for the vector-encoded pFVIII transgene. eGFP was visualized in live cells using an Olympus IX71.

Postnatal MSC Transplant

For the 1st transplant on animal #911, 30×10⁶ MSC, which had been subjected to a single round of transduction with the pFVIII lentivector and a single round of transduction with the eGFP lentivector, were resuspended in IMDM in a total volume of 5ml, and loaded into a 10ml syringe. Recombinant human FVIII was administered to restore #911's FVIII levels to 200% of normal and thus provide protection during the procedure. The animal was then sedated with an intravenous injection of Telazol (tiletamine/zolazepam), gently rolled onto his side, and his abdomen shaved. The peritoneal cavity was visualized with an Aloka veterinary ultrasound with a 5 MHz transducer. Following sterilization of the abdominal area with povidone-iodine, a 22-gauge needle was inserted through the abdominal wall and the cells were slowly injected into the peritoneal cavity under ultrasound visualization. The needle was withdrawn and pressure was applied to the site of injection for 2-3 minutes. The animal was then allowed to recover under close observation. For subsequent transplants on #911 and all transplants on #905, an identical procedure was followed, but the MSC were

subjected to 2 additional rounds of transduction with the pFVIII lentivector. Cell numbers were also increased (Tables 1/2) in an effort to improve therapeutic efficacy.

Immunofluorescence

Formalin-fixed, paraffin-embedded tissues were sectioned at 4 μ m, mounted on VistaVision Histobond slides (VWR), and incubated at 60°C for 45min. Synovial tissue was de-calcified prior to sectioning. For staining, sections were dewaxed in xylene, rehydrated, and rinsed in deionized water. Slides were then washed in PBS, followed by blocking in PBS+10% normal goat serum (NGS, Atlanta Biologicals) for 30min. Primary antibodies (rabbit anti-GFP, Abcam, Cambridge, MA; and mouse anti-FVIII, AbD Serotec, Raleigh, NC) were then added, diluted in 1mL PBS+2% NGS. After overnight incubation, slides were washed in PBS+2% NGS and stained for 30 min at room temperature with Alexa Fluor®-conjugated secondary antibodies (Invitrogen) diluted 1:400 in PBS+2% NGS. Slides were rinsed in PBS, counterstained with DAPI (Biogenex, Fremont, CA) and mounted using Cytoseal 60 (Thermo Scientific, Dubuque, IA). Slides were imaged using a Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan). Images were taken as z-stacks with 10-12 slices per image and then projected as 2-D images before being saved and processed using Adobe Photoshop. Sections from each tissue stained with secondary antibody alone served as negative controls in all experiments.

DNA Extraction and PCR

DNA was extracted from sheep tissues using the DNeasy Blood and Tissue kit according to manufacturer's instructions (Qiagen, Valencia, CA). 40ng of DNA was used for nested PCR with eGFP- or pFVIII-specific primers. The primers for the pFVIII transgene were designed by Blast-aligning porcine FVIII and ovine FVIII and selecting regions with disparate sequences to ensure the endogenous ovine FVIII gene would not be amplified. Extensive testing with normal sheep DNA confirmed the absolute specificity of these primers for the vector-encoded porcine FVIII transgene. The primary PCR was run for 30 cycles, after which 1 μ L of product was used for 30 cycles of nested PCR. Following an initial denaturation for 4 min at 95°C, each PCR consisted of 30 cycles of: 30sec denaturation at 94°C, 30sec-1min annealing at 57-60°C (dependent upon primers), and 30sec extension at 72°C; followed by 10min final extension at 72°C. Primers were as follows:

GFP primary (Anneal- 60°C):	FWD- 5'-ATGGTGAGCAAGGGCGAGGA
	REV- 5'-GCCGTTCTTCTGCTTGTCGGC
GFP nested (Anneal- 60°C):	FWD- 5'-CACCTACGGCAAGCTGACCC
	REV- 5'-CCCTTCAGCTCGATGCGGTT
pFVIII primary (Anneal 57°C):	FWD- 5'-GTGGTCTTCCGGGAATTGTC
	REV- 5'-GCTGGACGAAGTTCTGTCTG
pFVIII nested (Anneal- 60°C):	FWD- 5'-TGACGGCTCCTTCACGCA
	REV- 5'-AGGTTCTGCCCTTGCTCC

PCR products were analyzed on 1% agarose gels. DNA extracted from transduced MSC served as a positive control, while DNA from non-transduced sheep MSC served as a negative control.

Results

Clinical course prior to transplantation

Animal #911 was born on 9/24/2009, and was immediately diagnosed with HA upon observing prolonged dripping blood from the umbilical cord, a hallmark symptom in these

animals [20]. This diagnosis was confirmed by prolongation of his PTT and by performing a PCR-based RFLP for the FVIII mutation as we have previously described [20]. Over the next 3.5 months of life, he received 22 human FVIII (hFVIII) infusions (roughly 2 treatments per week) for multiple hematomas, rectal bleeding, and chronic, progressive, debilitating hemarthroses which had resulted in severe defects in posture and gait, rendering him nearly immobile. These events and the hFVIII treatment regimen appear in Table 1. By the end of December 2009, despite having received a total of 28,189U of hFVIII, his clinical condition had worsened to the point that euthanasia for humane reasons seemed inevitable.

Animal #905 was born on 9/20/2009, and was also diagnosed with severe HA immediately upon parturition, as detailed for animal #911. Animal #905's clinical/treatment history appears in Table 2. Animal #905 exhibited a markedly more sedentary/docile disposition than animal #911, which likely explains the reduced severity of his phenotype compared to that of #911, as has been described in human siblings with identical FVIII mutations [23]. Nevertheless, #905 still suffered a variety of spontaneous bleeding events requiring 8 hFVIII administrations during his first 4.5 months of life. These bleeds had also led to chronic debilitating hemarthroses of the leg joints, just as had occurred in #911. In addition, animal #905 developed a hematoma of the head which we feared could prove life-threatening if not completely resolved.

Choosing a Haploidentical Donor

In an ideal situation, one would use autologous cells to deliver a FVIII transgene, and thus avoid any complications due to MHC-mismatching. Unfortunately, the severe life-threatening phenotype of the HA sheep prevented us from collecting bone marrow (BM) aspirates to isolate autologous cells. We therefore elected to utilize haploidentical allogeneic cells from the rams that had sired the two HA lambs. Since multiple rams were utilized for breeding to the HA carrier ewes, we first performed ovine leukocyte antigen (OLA) typing on the HA lambs and the candidate rams to establish paternity and confirm haploidentity, and thereby minimize the potential for alloreactivity. PCR-based microsatellite OLA-typing was performed on DNA isolated from the candidate rams and from the two HA lambs, as detailed in the Materials and Methods section, and amplicon sizes for each allele compared. As shown in Supplementary Figure 1, this approach allowed us to unequivocally establish that the same ram had sired both HA animals (#911 and #905) and confirm haploidentity at all 5 of the microsatellite loci we tested within the OLA region of the sheep genome.

MSC Preparation, Transduction, and pFVIII Production

Based on our prior work in the fetal sheep model, we knew that the intraperitoneal transplantation of MSC results in widespread engraftment throughout all of the major organs [8-12, 24] and durable expression of vector-encoded genes [10, 12, 25], at least in the context of the developing fetus. Coupling this data with the documented immunosuppressive properties of MSC, we reasoned that it might be possible, in a postnatal setting, to use MSC as vehicles to deliver a FVIII transgene and achieve therapeutic benefit in HA. Therefore, after identifying the ram which had sired each HA lamb and could thus serve as a haploidentical donor, BM aspirates were collected and MSC isolated as detailed in Materials and Methods. We anticipated that, by using paternal (haploidentical) MSC, immunologic incompatibility between the donor and recipient should be minimized sufficiently to allow engraftment.

Following isolation, MSC were simultaneously transduced with 2 HIV-based lentivectors, the first of which encoded an expression/secretion optimized porcine FVIII (pFVIII) transgene, and the second encoded an eGFP marker gene to facilitate tracking and identification of donor cells *in vivo*. Combining the 2 vectors in the same transduction

unexpectedly resulted in preferential transduction with the eGFP vector, such that only about 15% of the MSC were transduced with the pFVIII-encoding vector, as assessed by qPCR. However, production of FVIII by the transduced MSC was still $\sim 1 \text{ unit}/10^6 \text{ MSC}/24\text{hr}$, as determined by chromogenic assay.

In an effort to enhance pFVIII production levels, we performed 2 additional rounds of transduction with only the pFVIII vector and then reassessed transduction efficiency and pFVIII production. These additional rounds of transduction yielded a population of MSC $>95\%$ of which were expressing the vector-encoded pFVIII transgene, as can be seen in Supplementary Figure 2, which is a representative immunofluorescence staining of the transduced MSC using an antibody to FVIII (red staining). This enhanced transduction successfully raised FVIII production levels to $\sim 6 \text{ units}/10^6 \text{ MSC}/24\text{hr}$.

Transplantation and Clinical Course

Animal #911—Given the rapidly deteriorating condition of animal #911, we elected to proceed with a transplant on January 6th (15 weeks of age), despite the fact that the paternal MSC had only undergone a single round of transduction, and were thus expressing fairly low levels of pFVIII. Just prior to transplant, the animal was treated with a dose of hFVIII calculated to correct his levels to 200%, to ensure no procedure-related bleeding occurred. He was then sedated, and 30×10^6 of the transduced MSC were then transplanted into the peritoneal cavity under ultrasound guidance in the absence of any preconditioning, as detailed in the Materials and Methods.

Following transplantation, FVIII activity (as assessed by chromogenic assay) was undetectable in the circulation, but #911's clinical picture improved dramatically. All spontaneous bleeding events ceased, and he enjoyed an event-free clinical course, devoid of spontaneous bleeds, obviating the need for hFVIII infusions (Table 1, Figure 1A). Existing hemarthroses resolved, the animal's joints recovered fully and resumed normal appearance (as assessed by two attending veterinarians), and he regained normal posture and gait, resuming a normal activity level. To our knowledge, this represents the first report of phenotypic correction of severe HA in a large animal model following transplantation of cells engineered to produce FVIII, and the first time that reversal of chronic debilitating hemarthroses has been achieved.

Animal #905—Based on the remarkable clinical improvement we had achieved in animal #911, and to avoid complications due to an existent head hematoma, on February 9, 2010 (18 weeks of age), we transplanted animal #905 with 120×10^6 paternal MSC which had been subjected to 1 round of transduction with the eGFP vector and 3 rounds of transduction with the pFVIII vector ($>95\%$ transduced and expressing FVIII). We used the same approach as we had employed with animal #911, administering the cells via the peritoneal cavity under ultrasound guidance in the absence of any preconditioning. We anticipated that by transplanting 4x's the number of cells with roughly 6x's the transduction efficiency, we would achieve pronounced improvement and therapeutic levels of FVIII in the circulation of this animal, given that the transplanted cells were producing ~ 24 x's the levels of FVIII (720units/24hrs) as those transplanted into #911. In similarity to animal #911, hemarthroses present in #905 at the time of transplant resolved, and he resumed normal activity shortly after transplantation. Like #911, this second animal also became factor-independent following the transplant (Table 2, Figure 1B). These results thus confirm the ability of this MSC-based approach to provide phenotypic correction in this large animal HA model. However, just as we had observed in animal #911, no FVIII was detectable in the circulation of this animal, making the mechanism by which this procedure produced such pronounced clinical improvement indeterminate.

Inhibitor Analysis

Despite the pronounced clinical improvement we observed in animal #911, this animal mounted a rapid and fairly robust immune response to FVIII, in similarity to prior studies performed with HA mice [22]. Before transplant, #911 had Bethesda titers against hFVIII of only ~3, yet this lifesaving procedure resulted in a rise in Bethesda titer to ~800 against the vector-encoded pFVIII and nearly 700 to hFVIII (Supplementary Figure 3A). The formation of such high titer inhibitors with cross-reactivity to the human protein is surprising, given the well established ability to successfully use porcine FVIII products in human patients to bypass existing anti-hFVIII inhibitors [26-29]. Similarly, despite having no detectable inhibitors prior to transplant, animal #905 developed titers of ~150 Bethesda units against the vector-encoded pFVIII following transplantation (Supplementary Figure 3B) which, like those in #911, exhibited cross-reactivity to the human protein.

At 2 months after the 1st transplant (5.5 months of age), #911 remained factor independent and stable, despite the continued presence of high titer FVIII inhibitors and the complete absence of any detectable FVIII activity in the circulation. Therefore, to test whether better transduction efficiency and higher cell numbers would result in corrective circulating levels of FVIII, we performed a 2nd transplant on this animal on 2/9/2010 using the MSC that had been subjected to 2 additional rounds of transduction with the pFVIII-encoding lentivector. We also hypothesized that delivering a larger dose of gene-modified cells, with its correspondingly higher level of steady FVIII production could, in effect, function as a cellular-based immune tolerance induction (ITI) procedure and thereby break the high titer inhibitors which had developed consequent to the 1st transplant [2, 30]. We used an identical transplant procedure, except for this 2nd transplant, we transplanted 125×10^6 MSC, >95% of which were expressing pFVIII as assessed by immunofluorescence staining (Supplementary Figure 2). This 2nd transplant thus delivered ~24x's the levels of FVIII (~720units/24hrs) of the first transplant. Nevertheless, FVIII activity was still not detected in the circulation post-transplantation, and the Bethesda titers remained largely unchanged, even following a course of prednisone (Supplementary Figure 4), suggesting that this approach, despite its clinical efficacy, will require further optimization prior to use for ITI. After a remarkably event-free clinical course, animal #911 had to be euthanized at 14wks after his 1st transplant, after ingesting a sharp object which caused a massive hematoma of the larynx, reducing his airway by >75%.

While a 2nd transplant did not induce tolerance in #911, we reasoned that the substantially lower titers of the inhibitors in animal #905 might prove easier to overcome than those in animal #911. We therefore performed a 2nd transplant with 60×10^6 MSC at 2 months post-1st transplant in this animal to attempt to break the inhibitors and achieve detectable levels of FVIII activity in the circulation. Following this 2nd transplant, #905 continued his factor-independence and exhibited no spontaneous bleeds. However, FVIII activity was still undetectable in his circulation and the inhibitor titers continued unabated. A 3rd transplant at 3.25 months after the 2nd transplant (5.25 months after the 1st transplant), using 31.5×10^6 MSC also failed to reduce the inhibitor titer (remained ~150 Bethesda units) or produce detectable levels of FVIII activity within #905's circulation, but #905's clinical picture remained unchanged. Unfortunately at roughly 1 month after this 3rd transplant, a group of near-term pregnant ewes were brought into the lambing barn in which #905 was being housed. This resulted in a dramatic change in his disposition, and his newly agitated/excited state resulted in several accidental traumas which precipitated bleeding events within his leg joints which progressively worsened despite providing human FVIII infusions and a course of prednisone and Tylenol. As a result, we were forced to euthanize this animal at roughly 1 year of age (7.5 months post-1st transplant).

Post-Mortem Analysis on Animal #911

Following euthanasia of animal #911, we performed a detailed analysis of this animal's tissues to begin deciphering the mechanism whereby this novel MSC-based gene delivery produced its pronounced therapeutic effect at a systemic level. PCR analysis using primers to the vector-encoded pFVIII transgene and to eGFP both demonstrated readily detectable levels of MSC engraftment in nearly all tissues analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus (Figure 2), but not in diaphragm. The absence of amplification within the synovial sample was determined to be due to the absence of cells in the synovial fluid sample used for PCR analysis, as amplification with primers for G3PDH also failed to generate a PCR product. Importantly, these molecular analyses thereby established that it is possible to achieve widespread durable engraftment of MSC following transplantation in a postnatal setting in a large animal model without the need for preconditioning/ablation, and in the absence of any selective advantage for the donor cells.

To understand how the MSC were producing the observed systemic improvements following engraftment in these diverse sites, we next performed confocal immunofluorescence analysis on frozen tissue sections. As can be seen in Figure 3A, large numbers of GFP+ MSC are present within the synovium of the joints which exhibited hemarthrosis at the time of the 1st transplant, and these cells are expressing FVIII (Figure 3B, red) suggesting the transplanted MSC possessed the intrinsic ability to home to sites of ongoing injury/inflammation and persisted for over 3 months, releasing FVIII locally within the joint, providing an explanation for the dramatic improvement we observed in this animal's joints. This finding is in agreement with prior studies by Paul Monahan's and Jude Samulski's groups [31], showing that local delivery of FIX-AAV to the joints of mice with injury-induced hemarthroses led to resolution of the hemarthroses in the absence of any detectable FIX in the circulation. While this finding provides an explanation for the reversal of the joint pathology present in this animal at transplant, it cannot explain the observed systemic benefits such as the cessation of spontaneous bleeding events.

Confocal analysis (Figure 4) of the small intestine also revealed engrafted cells, albeit at relatively low levels, demonstrating that MSC can still engraft within the intestine following postnatal transplantation, just as we had observed in our prior studies in fetal recipients [12]. Given the ease with which proteins secreted from cells within the intestine can enter the circulation, future studies aimed at improving the levels of engraftment within the intestine have the potential to greatly improve the systemic release of FVIII.

In addition to the intestine and hemarthrotic joints, significant levels of engraftment were also seen within the thymus of this animal (Figure 5). While the ability of the transplanted MSC to traffic to the thymus could clearly have important implications for the likelihood of long-term correction with this approach to HA treatment, additional studies are required to determine with which cells within the thymus these MSC are interacting to ascertain the immunologic ramifications of thymic engraftment.

Discussion

Current HA treatments allow many patients with hemophilia to live relatively normal lives, but their high cost, the need for lifelong therapy, and the inaccessibility of these therapies to the vast majority of patients with hemophilia worldwide [32] highlight the urgent need to develop novel HA therapies offering longer-lasting benefit or permanent cure [3-6].

We recently re-established a line of sheep with a naturally-occurring frame-shift/premature stop codon mutation within the FVIII gene. These sheep accurately recapitulate the clinical symptoms present in human patients with severe HA. Importantly, these sheep all

spontaneously develop severe, debilitating hemarthroses, making them unique among the animal models of HA. In addition to the accuracy of their clinical presentation of HA, sheep share many important physiological, developmental, and immunological characteristics with humans [8, 33-35], making them a good preclinical large animal model for developing/testing stem cell and/or gene therapy-based treatments for HA and obtaining results of high clinical significance.

In the present studies, we used this line of sheep to test a novel approach to treating HA in which MSC served as delivery vehicles for a pFVIII transgene. This approach was technically straightforward, involving a single injection into the peritoneal cavity under ultrasound guidance, in the absence of any prior cytoablation/conditioning, and thus posed minimal risk to the recipient. The MSC transplant successfully converted both animals from a severe, life-threatening phenotype to a moderate phenotype, devoid of spontaneous bleeds, but still bleeding in response to accidental trauma. Remarkably, this approach also resulted in a complete reversal of the joint stiffness/swelling as well as the gait and postural defects that had developed as a result of frequent hemarthroses during the first months of life. To our knowledge, this represents the first time that reversal of the severe, crippling hemarthroses which plague human patients with hemophilia A has been achieved. It is important to note that although current protein replacement therapy given on-demand prevents life-threatening bleeds, it does not prevent microhemorrhaging within the joints. Moreover, unless administered immediately at the first sign that factor is needed, major bleeds into the joints can still occur. Over time, this leads to the development of chronic hemarthroses that progressively worsen with age, causing pain and decreased mobility, and greatly reducing quality of life. The present report thus opens the door to the development of new MSC-based therapies for this debilitating condition, either systemically or, perhaps, administered directly into affected joints of hemophilia patients.

Despite the marked clinical success we achieved, however, both animals in these studies developed high-titer inhibitors to the vector-encoded pFVIII, which also recognized human FVIII. While the reason for these high titer inhibitors is not presently known, several possible explanations exist. The first of these relates to the use of a pFVIII transgene which, by virtue of its xenogeneic origin, may be inherently immunogenic in sheep. This was perhaps further confounded by the treatment of both animals with human FVIII prior to receiving the transplant. Given the successful clinical use of pFVIII in human patients who harbor inhibitors to human FVIII [26-29], it is unlikely that the porcine origin of the transgene is the sole/major causative factor in the formation of high titer inhibitors. Nevertheless, the only way to eliminate this possibility will be to perform studies employing cells which have been transduced with a lentivector encoding ovine FVIII, rather than the pFVIII construct we utilized in the present studies. We are currently developing a vector to address this issue.

Looking beyond the immunogenicity of FVIII itself, evidence from recent studies in both hemophilia patients [36, 37] and in basic immunology [38-40] strongly implicates the environmental, or clinical, context in which an antigen is presented to the host's immune system as critical in determining how the immune system responds to this new information [38-41]. In the context of HA, if FVIII is only administered when the patient is experiencing a serious or life-threatening bleed, the immune system might associate FVIII with the presence of inflammatory cytokines and other danger signals. If, on the other hand, FVIII were administered prophylactically, during periods of homeostasis, inhibitor formation might be less likely [42]. This is particularly salient when evaluating the results of the present studies. Sheep #911, who had been treated with hFVIII with high frequency for numerous life-threatening bleeds and received his 1st transplant at a time when his clinical picture had become so severe/debilitating that euthanasia seemed unavoidable, formed 4-5-

fold higher titer inhibitors than did sheep #905, who had experienced fewer bleeds and was relatively clinically stable at the time of his 1st transplant.

In addition to the clinical context in which FVIII is presented, the peritoneum is rather unique from an immunological standpoint. Following delivery to the peritoneum, prior research suggests that the majority of the cells are either engulfed by the omentum or they traffic to the circulation via the lymphatics of the abdomen [43]. Although the precise functions of the omentum are not yet fully defined, recent studies have indicated that it engulfs foreign entities within the peritoneum [44-48], whereupon resident dendritic cells (DC) present any ingested foreign antigens to local T cells, priming both a T and B cell response. It is therefore reasonable to speculate that one of the factors contributing to the robust inhibitor formation we observed following IP transplantation of FVIII-expressing MSC is the uptake of these cells by the omentum with subsequent presentation of FVIII by resident DC. However, a prior clinical trial showed that omental implantation of autologous skin fibroblasts transfected with a hFVIII-expressing plasmid resulted in clinical improvement for up to 10 months in one patient without inducing inhibitors [49]. In light of this prior study, it is thus likely that the induction of inhibitors in our present study was not due, per se, to the uptake of the transplanted cells by the omentum, but rather, resulted from the presentation of multiple foreign antigens to DC residing within the omentum following the delivery of a xenogeneic FVIII transgene in the context of a haploidentical cell carrier. Given the durable engraftment/persistence of the transplanted MSC within multiple organs of the recipients, it does not seem that a very robust cell-mediated immune reaction was induced to the paternal cells, but only future studies with autologous MSC will allow this issue to be addressed definitively.

The observation of engraftment of MSC within the small intestine of animal #911 is particularly important in this respect, since the intestine represents a major immune organ in which several specialized lymphoid structures and immune cell types reside [50]. Under normal physiologic conditions, in the absence of inflammation or other danger signals, intestinal-resident DC are unable to undergo maturation before presenting enteric antigens to naïve lymphocytes. Therefore Tregs are generated and tolerance to the antigen develops. It is thus possible that further optimized delivery of the FVIII-expressing MSC to the intestine could promote the induction of immune tolerance, rather than immunity, to the vector-encoded FVIII.

Likewise, the demonstration that IP transplanted MSC traffic to the thymus and engraft could have important implications for the development of strategies to avoid the observed inhibitors. Detailed analyses of the thymus from both animals are currently ongoing to ascertain with which thymic cells the MSC are interacting, in an effort to glean clues to the immunologic consequences of thymic engraftment.

Regardless of the mechanism by which high-titer inhibitors formed in the 2 animals which received this procedure thus far, their presence raises the possibility that we did, in fact, achieve therapeutic levels of FVIII, but its activity was simply being neutralized by these inhibitors, accounting for the negative results by chromogenic assay. Given FVIII's ability to exhibit so-called race kinetics, the FVIII produced by the engrafted cells may be entering the circulation and "racing" to the sites of bleeds prior to being bound/inactivated by inhibitors, yet would go undetected in the chromogenic assay. This would explain the marked phenotypic improvement seen in these animals, and the cessation of spontaneous bleeds in the absence of detectable plasma FVIII activity.

In summary, in the present studies, we describe a novel postnatal, non-ablative MSC transplantation procedure which successfully converted 2 HA animals from a severe, life-

threatening phenotype to a moderate phenotype, devoid of spontaneous bleeds, and completely reversed the crippling hemarthroses present at the time of treatment. To our knowledge, this is the first time such a dramatic improvement in clinical picture and phenotype has been achieved in a large animal model of HA following stem cell-based gene therapy, and our findings could thus pave the way for treating hemophilia patients with debilitating hemarthroses. Further mechanistic studies will be required to understand and ultimately overcome the formation of inhibitors resultant from this procedure since, in their absence, this approach could result in even more pronounced clinical improvement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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PCR-based Microsatellite OLA Typing

To establish paternity and assess MHC compatibility, peripheral blood was collected from the rams which were bred to the HA carrier ewes, and DNA was extracted according to manufacturer's instructions with a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), followed by quantitation at the Nevada Genomics Center. 20ng of DNA was then used to perform multiplex PCR with primers designed to amplify microsatellites present within the ovine leukocyte antigen (OLA) region of the sheep genome. Primers were chosen after extensive testing on sheep of known pedigree to confirm their ability to amplify products of differing sizes from these microsatellite regions in unrelated sheep, and thus provide a rigorous means of OLA typing. PCR was performed using the QIAGEN Multiplex PCR Master Mix, fluorescently labeled primer mix at a final concentration of 2nM, and DNA. Fluorescently labeled primers contained one of four 3' end-labeled dyes: VIC, NED, PET, or 6-FAM (Applied Biosystems, Carlsbad, CA). PCR cycling conditions were as follows: Initial melting phase of 15 min at 94°C (to deactivate chemical Taq inhibitor) followed by 34 cycles of 94°C for 30 sec, 55-57°C for 1 min 30 sec, and 72°C for 30 sec; this was followed by a final extension for 30 min at 62°C. The PCR products were then subjected to fragment analysis by capillary electrophoresis on an Applied Biosystems 3730 Genetic Analyzer along with an internal size standard (GeneScan™ LIZ® GS500 size standard, Applied Biosystems). Resultant PCR products were then analyzed using GeneMapper v3.7 (Applied Biosystems) software in order to determine sizing quality, binning of microsatellite alleles, and consolidation of microsatellite alleles for each animal. DNA extracted from the peripheral blood of the HA sheep (#911 and 905) was amplified using the identical primers/PCR conditions, and the amplicons obtained with each primer set were then compared to those obtained with the candidate rams to establish paternity and confirm a haploidentical match was present.

Figure 1.
(A) Cumulative Units of FVIII Administered for Spontaneous Bleeding Events in Animal #911. To assess the therapeutic benefit of the transplantation of pFVIII-expressing MSC, we calculated the cumulative dose of FVIII administered to the animals over time. Displayed in this Figure are the results of this analysis for animal #911. Note: FVIII administered following accidental trauma is not included in the presented calculation. **(B) Cumulative Units of FVIII Administered for Spontaneous Bleeding Events in Animal #905.** To assess the therapeutic benefit of the transplantation of pFVIII-expressing MSC, we calculated the cumulative dose of FVIII administered to the animals over time. Displayed in this Figure are the results of this analysis for animal #905. Note: FVIII administered following accidental trauma is not included in the presented calculation.

Figure 2. PCR Analysis of Tissues from Animal #911

Following collection of the tissues from animal #911, DNA was isolated and analyzed by PCR using primers specific to the vector-encoded eGFP transgene. As can be seen, MSC lodged/engrafted within nearly all tissues examined, with the exception of the diaphragm. The synovium also failed to amplify, but this was due to lack of cells in the synovial fluid sample used for DNA isolation.

Figure 3. Engraftment of MSC in Synovium of Animal #911 Following Postnatal Intraperitoneal Transplantation

Frozen sections prepared from the synovium of the hemarthrotic joints of animal #911 were visualized directly for GFP fluorescence (Figure 5A) or stained with an antibody to FVIII (Figure 5B; red cells are FVIII+) to assess the ability of the transplanted MSC to traffic to sites of active injury/inflammation and locally release FVIII.

Figure 4. Engraftment of MSC in Small Intestine of Animal #911

Frozen sections prepared from the small intestine of animal #911 were visualized directly for GFP fluorescence to assess the engraftment of the transplanted MSC within this tissue. Shown here are two representative sections from the small intestine. Insets are magnified views of regions containing GFP+ positive cells.

Figure 5. Engraftment of MSC in Thymus of Animal #911

Frozen sections prepared from the thymus of animal #911 were visualized directly for GFP fluorescence to assess the engraftment of the transplanted MSC within this tissue. Shown here is a representative section from the thymus. As can be seen, numerous MSC have engrafted within this tissue. The boxed inset is a magnified view of one of the regions containing GFP+ positive cells.

Table 1**Animal #911's Clinical Course and Treatment Schedule**

Date	F8 dosage	Type	Reason
9/24/2009	295U (100%)	Koate	dripping blood from umbilical cord
9/29/2009	318U (100%)	Koate	Limping on left rear leg
10/12/2009	472U (100%)	Koate	Limping on right front leg
10/13/2009	472U (100%)	Koate	Limping on right front leg
10/16/2009	472U (100%)	Koate	Limping on right front leg
10/28/2009	684U (100%)	Koate	Limping on rear legs
10/30/2009	684U (100%)	Koate	Limping on rear legs
11/3/2009	682U (100%)	Koate	Limping on right front leg
11/4/2009	590U (87%)	Koate	Limping on right front leg
11/16/2009	800U (100%)	Recombinant	Limping on right rear leg (swollen "elbow" joint)
11/19/2009	800U (100%)	Recombinant	Rectal Bleeding
	800U (100%)	Recombinant	Rectal Bleeding
	1000U (100%)	Recombinant	Rectal Bleeding
11/20/2009	1000U (100%)	Recombinant	Limping on right rear leg (swollen "elbow" joint)
12/2/2009	1000U (100%)	Recombinant	Limping on right rear leg
12/3/2009	1000U (100%)	Recombinant	Limping on right rear leg
12/8/2009	1000U (100%)	Recombinant	Limping on right rear leg
12/10/2009	1000U (100%)	Recombinant	Limping on left front leg; hematoma on jaw
	1000U (100%)	Recombinant	Limping on left front leg; hematoma on jaw
12/11/2009	2000U (200%)	Recombinant	Limping on left front leg; hematoma on jaw
12/21/2009	2000U (200%)	Recombinant	Limping on right rear leg
12/22/2009	2000U (200%)	Recombinant	Limping on right rear leg
12/29/2009	2000U (200%)	Recombinant	Limping on right rear leg + right front leg
	2000U (200%)	Recombinant	Limping on right rear leg + right front leg
12/30/2009	2120U (200%)	Recombinant	Limping on right rear leg + right front leg
12/31/2009	2000U (200%)	Koate	Limping on right rear leg + right front leg
1/6/2010	2000U (200%)	Recombinant	prior to MSC transplant
	2000U (200%)	Recombinant	following MSC transplant (30×10 ⁶ cells)
1/19/2010	1000U (~85%)	Recombinant Porcine	to cover after blood draw
2/9/2010	1000U (~85%)	Recombinant Porcine	to cover during 2nd MSC transplant (125×10 ⁶ cells)
2/22/2010	1100U (~90%)	Recombinant Porcine	limping on left front leg after trapping "knee" in fence
2/25/2010	1060U (100%)	Recombinant Porcine	Limping on left rear leg
	start 10 day course of prednisone		
3/11/2010	1550U (100%)	Recombinant Porcine	slight limp; both front legs a bit stiff
4/13/2010	2562U (~150%)	Recombinant Porcine	hematoma on left rear leg and favoring left front leg after moving to new pen
	start 10 day course of prednisone		

Date	F8 dosage	Type	Reason
4/16/2010	1750U (100%)	Recombinant Porcine	hematoma on left rear leg and favoring left front leg
4/18/2010	3071U (~150-200%)	Recombinant Porcine	hematoma of larynx + epiglottis after trauma; euthanized 4/20

Table 2

Animal #905's Clinical Course and Treatment Schedule

Date	F8 dosage	Type	Reason
9/20/2009	136U (100%)	Koate	dripping blood from umbilical cord
	136U (100%)	Koate	tail hematoma (mother stepped on tail)
10/5/2009	302U (100%)	Koate	Hunched appearance and dripping blood from banded tail
10/12/2009	354U (100%)	Koate	Limping on left rear leg
10/21/2009	460U (100%)	Koate	Limping on right front leg
10/30/2009	566U (100%)	Koate	Limping on front legs
1/8/2010	1000U (100%)	Recombinant Porcine	Limping on right rear leg
1/27/2010	1000U (100%)	Recombinant Porcine	Head hematoma
2/9/2010	1000U (100%)	Recombinant Porcine	Limping on right rear leg + coverage for 1st MSC transplant; 125×10 ⁶ cells
4/7/2010			2nd MSC transplant (60×10 ⁶ cells) to try to "break" inhibitors
7/12/2010			3rd MSC transplant (31.5×10 ⁶ cells) to try to "break" inhibitors
8/14/2010	1927U (100%)	Recombinant Human	lane (both rear legs) after bedding change & bringing pregnant ewes into barn
8/16/2010	start course of prednisone and tylenol		sitting "dog-style", won't put weight on rear legs
8/24/2010	2148U (100%)	Recombinant Human	same
9/2/2010	final prednisone dose		animal standing and walking fairly well
9/8/2010	2148U (100%)	Recombinant Human	rear legs lame again