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Expression in blood cells may contribute to biochemical and pathological improvements after neonatal intravenous gene therapy for mucopolysaccharidosis VII in dogs

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Abstract

Mucopolysaccharidosis VII (MPS VII) is a lysosomal storage disease due to deficient activity of β-glucuronidase (GUSB) that results in accumulation of glycosaminoglycans in many organs. We have previously reported that neonatal intravenous injection of a gamma retroviral vector (RV) expressing canine GUSB resulted in transduction of hepatocytes, high levels of GUSB modified with mannose 6-phosphate in blood, and reduction in disease manifestations in the heart, bone, and eye. However, it was unclear if liver was the only site of expression, and the effect upon other organs was not assessed. We demonstrate here that blood cells from these RV-treated MPS VII dogs had substantial copies of RV DNA, and expressed the RNA at 2% of the level found in liver. Therefore, expression of GUSB in blood cells may synergize with uptake of GUSB from blood to reduce storage in organs. The RV-treated dogs had marked biochemical and pathological evidence of reduction in storage in liver, thymus, spleen, small intestines, and lung, and partial reduction of storage in kidney tubules. The brain had 6% of normal GUSB activity, and biochemical and pathological evidence of reduction in storage in neurons and other cell types. Thus, this neonatal gene therapy approach is effective and might be used in humans if it proves to be safe. Both secretion of enzyme into blood by hepatocytes, and expression in blood cells that migrate into organs, may contribute to correction of disease.

Keywords: Gene therapy; Lysosomal storage disease; Mucopolysaccharidosis; Glycosaminoglycan; Retroviral vector

Introduction

The mucopolysaccharidoses (MPS) are lysosomal storage diseases (LSD) due to deficient activity in enzymes involved in degradation of glycosaminoglycans (GAGs) [1,2]. The overall incidence of MPS is ~1:29,000 live births [3]. Clinical manifestations are due to the accumulation of GAGs throughout the body, which disrupts the function of cells and organs. Features include hepatosplenomegaly, recurrent pulmonary infections, growth retardation, mobility problems, dysostosis multiplex, facial dysmorphia, visual and hearing defects, and cardiac valvular abnormalities. Mental retardation occurs for some, but not all, of these disorders.

Mucopolysaccharidoses VII (Sly syndrome; OMIM 253220) is due to deficient activity of the 300 kDa lysosomal enzyme β-glucuronidase (GUSB; EC 3.2.1.31) [4]. Although rare with an incidence of less than 1:1,000,000 live births,

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the existence of mouse [5], dog [6], and cat [7] models have made this a model system for studying the effects of novel therapies on MPS. In addition, a simple histochemical stain for the enzyme activity facilitates the analysis of organs to identify cells that express the enzyme or take it up from blood.

Some types of MPS are currently treated with hematopoietic stem cell transplantation (HSCT) or enzyme replacement therapy (ERT) (reviewed in [8]). HSCT has reduced the clinical manifestations in mice with MPS VII [9,10], and in humans with MPS VII [11] or MPS I [12]. One possible mechanism is migration of blood cells into organs, where they secrete enzyme modified with mannose 6-phosphate (M6P) that can be taken up by adjacent cells via the M6P receptor (M6PR) present on their surface [13]. HSCT is limited by the need for a compatible donor, and the risks and costs of the procedure. ERT involves the intravenous (IV) injection of enzyme that has M6P, which can diffuse into organs and be internalized via the M6PR [14]. ERT has been therapeutic in mice with MPS VII [8,15], and in humans with MPS I [16]. Difficulties with ERT include the need for frequent infusions and the high cost of the recombinant protein. For example, ERT for MPS I involves injection once a week and costs over $10,000 per kg per year.

Gene therapy could be used to treat the clinical manifestations of MPS [17]. This could involve transduction of a patient's HSC, whose progeny could migrate into other organs and correct disease in a fashion analogous to HSCT. Gene therapy of hematopoietic cells has improved manifestations in mice with MPS VII [18,19], but has not been effective in large animals with MPS [20,21]. Alternatively, other organs or cells could be modified to secrete enzyme with M6P into the blood, and enzyme in blood could be taken up by cells in other organs via the M6PR. Indeed, liver, fibroblasts, and muscle have all been transduced with viral or plasmid vectors in an attempt to modify cells to secrete enzyme into blood (reviewed in [8]). Expression from livers of mice from adeno-associated virus (AAV) vectors [22,23], adenoviral vectors [24], or retroviral vectors (RVs) [25,26] have resulted in the highest levels of enzyme in serum and the most-profound effect upon disease manifestations.

Although a variety of HSCT- and liver-directed gene therapy approaches have been effective in mice with MPS VII [8], few studies have been performed in large animals. Results in large animals with a long lifespan will likely be more predictive of effects in humans than results in mice. MPS VII dogs are homozygous for an arginine to histidine substitution at amino acid 166 in the canine GUSB (cGUSB) protein [27]. Features in MPS VII dogs resemble those in humans, except mental retardation is difficult to assess, and hepatosplenomegaly is less severe. Implantation of genetically modified fibroblasts into MPS VII dogs resulted in uptake of GUSB and reduction in lysosomal storage in liver, but serum levels were low and there was no effect on other organs [28].

We have previously demonstrated that neonatal IV injection of an amphotropic gamma RV into newborn MPS VII dogs resulted in a remarkable improvement in bone and joint disease, corneal clouding, and heart disease [29–31]. This was believed to be due to transduction of hepatocytes that secreted enzyme into blood for the following reasons: (1) hepatocytes in transduced dogs had high enzyme activity at 4 months after transduction [32]; (2) organs other than liver and spleen had very few RV DNA copies, and did not express an RV at 1 week after transduction of newborn dogs with a β-galactosidase-expressing vector [32]; and (3) RV RNA levels in liver of MPS VII mice that were transduced with a similar neonatal gene therapy procedure were >100-fold that found in spleen or other organs at 6 months after transduction [26]. However, we recently determined that there was substantial transduction of hematopoietic cells in mice after neonatal transduction [33], raising the possibility that transduction of hematopoietic cells could contribute to correction of disease in dogs. It will be important to evaluate the biodistribution and expression of the vector to determine if correction of disease is indeed due to secretion of enzyme into blood from the liver, or to transduction of cells in other organs. In addition, the effect of this gene therapy approach upon lysosomal storage in other organs has not yet been reported. We demonstrate here that there is expression from the RV in spleen and peripheral WBCs, which suggests that expression in these sites might contribute to correction of disease. We also demonstrate the effect of the gene therapy procedure on biochemical and pathological manifestations of MPS VII in organs that have not been evaluated previously.

Materials and methods

Reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Analysis of animals

Mucopolysaccharidoses VII dogs were transduced with IV injection of hAAT-cGUSB-WPRE at 2–3 days after birth, as described previously [33]. Controls were normal or MPS VII dogs that did not receive RV. Some dogs were sacrificed at 6–7 months after birth using 80 mg/kg of sodium pentobarbital (Veterinary Laboratories, Lenexa, KA). Dog M1339 died while under propofol anesthesia during removal of cerebrospinal fluid. He appeared to be in good health prior to the procedure. Dogs were perfused with two liters of cold saline prior to collection of tissues.

Analysis of GUSB and β-Hex activity

Organs were homogenized with 2 μl homogenization buffer per milligram of tissue and tested for GUSB and total β-hexosaminodase (β-Hex) activity as described [26,34]. One unit of enzyme produces 1nanomole of product in 1 h. The

protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). For analysis of peripheral white blood cells (WBC), 3 ml of blood was mixed with 9 ml of Puregene RBC lysis solution (Gentra Systems, Minneapolis, MN), washed, and resuspended in homogenization buffer.

GAG assay

Glycosaminoglycan levels were determined in the same homogenates that were used for enzyme assays using a sulfated glycosaminoglycan kit from Blyscan (Newtownabbey, N. Ireland). Unless otherwise stated, samples containing 150 μg or less extract protein in 20 μl homogenization buffer were mixed with 30 μl water and 500 μl dye reagent at room temperature for 30 min. Samples were centrifuged at 12,000*g* for 10 min, the supernatant was poured off and any remaining drops absorbed with paper, and the pellets were resuspended in 250 μl dissociation reagent by vortexing for 1 min. Two hundred microliters was placed into a 96-well ELISA plate and the optical density (OD) at 655 nm was read. Standards were prepared with chondroitin 4-sulfate processed in the same way. Urine GAG levels were normalized to creatinine levels, which were determined using a kit from Sigma Chemical.

Histochemical stain for GUSB activity and histopathology

Organs were embedded in optimal cutting compound (OCT; Sakura Finetek USA, Torrance, CA) and 8 μm sections were stained overnight for GUSB activity with 0.25 mM naphthol AS-BI-glucuronide as described [34]. For analysis of peripheral WBCs, blood was mixed with three volumes of Puregene RBC lysis solution (Gentra Systems), incubated at room temperature for 5–10 min, centrifuged for 5 min at 2000*g*, and the cells were washed with PBS. Cells were fixed to eight-well slides and stained for GUSB activity. For histopathology, organs were fixed with 2% glutaraldehyde and 4% paraformaldehyde in PBS, embedded in Epon (Electron Microscopy Sciences, Fort Washington PA), and 1 μm sections were stained with toluidine blue (TB). Pathology was evaluated without knowledge of the genotype and treatment status.

Evaluation of DNA and RNA

For real-time PCR of DNA from organs or peripheral WBCs, 0.1–0.5 g pieces of organ, or cells from 10 ml heparinized blood, were homogenized in guanidinium, and DNA and RNA were extracted [32]. To remove small molecular weight nucleic acids, ~20 μg DNA was bound to beads from the QIAEX II gel Extraction Kit (Qiagen, Valencia, CA) and eluted according to the manufacturer's instructions. DNA was used for real-time PCR with Taqman technology (Applied Biosystems, Rockville, MD) and primers specific for the WPRE of the RV to determine the RV DNA copy number [26], with normalization to the dog β-actin sequence [32]. Standards were DNA from hAAT-cGUSB-WPRE-transduced cells diluted with DNA from liver of a non-transduced dog. DNA from blood cells was also evaluated in a competitive PCR at some times. Genomic DNA was isolated from whole blood with Generation Capture Columns (Gentra Systems, Minneapolis, MN). PlatinumTaq (Invitrogen, Carlsbad, CA) was used for PCR in a solution containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphates, and 0.5 μM of each primer. PCR primers from exon 4 (5′-CCCTGTGCTCCTCTACACCAC-3′) and exon 5 (5′-CACGACCTTGCCTTCCTCATC-3′) of the cGUSB gene resulted in a 621-bp product from the genomic GUSB sequence, and a 163-bp product for the RV sequence that lacked an intron. PCR products were separated on 8% nondenaturing polyacrylamide gel electrophoresis and stained with ethidium bromide.

For analysis of RNA from organs and peripheral WBCs by real-time reverse transcriptase (RT)-PCR, approximately 1 μg of RNA was reverse transcribed according to the manufacturer's instructions in a 20 μl reaction volume with the 3′WPRE and the 3′ β-actin primers that were used for the real-time PCR, and a Superscript III kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed on 2 μl sample with Taqman technology and normalization to the β-actin signal. For RT-PCR with gel electrophoresis of RNA from peripheral WBC, RNA was isolated from whole blood with the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA). RT was performed and products were amplified with primers from exon 12 of the cGUSB cDNA (5′-CTTCACTCGCCAGAGACAAC-3′) and the WPRE (5′-AAGCCATACGGGAAGCAATAG-3′) sequences to give a 520 bp product. A 702 bp canine β-actin control was amplified with the primers 5′-TGACCCAGATCATGTTT GAGACC-3′ and 5′-TCCTGCTTGCTGATCCACATC T-3′.

Statistical evaluations

Averages ± the standard deviation (SD) were calculated for all values. The program SigmaStat was used to determine the statistical significance of differences between groups using one way analysis of variation (ANOVA) with Tukey post hoc analysis.

Results

The transduction of "RV-treated" MPS VII dogs with the IV injection of the amphotropic gamma RV designated hAAT-cGUSB-WPRE at 3 days after birth was reported previously [32]. This RV contained the liver-specific human α1-antitrypsin promoter (hAAT), the canine GUSB cDNA, and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Five RV-treated MPS VII dogs achieved transduction of ~2% of hepatocytes as determined by histochemical staining of liver biopsy samples, and an average serum GUSB activity of 195 ± 142 U/ml

(SD), which is 73% of normal. Three of the RV-treated dogs (M1328, M1332, and M1337) are doing well with stable levels of serum GUSB activity at 4.5 years after transduction, and were used for the analyses of peripheral WBCs and urine shown in this study. Two of the RV-treated MPS VII dogs were sacrificed at 6–7 months after birth and were used for the analyses of other organs: M1312 and M1339 had 166 U/ml (62% of normal) and 132 U/ml (49% of normal) of serum GUSB activity, respectively, at the time of sacrifice. An additional "HGF/RV-treated" dog (M1287) that received hepatocyte growth factor (HGF) prior to the infusion of a 4-fold higher dose of RV achieved stable expression of 18,000 U/ml (67-fold normal) of GUSB activity in serum, and was used for the analysis of peripheral WBCs here.

Distribution of RV DNA in organs

The site of gene transfer is important to identify the mechanism by which correction of disease occurs. Therefore, DNA was isolated from organs at 6–7 months after transduction and analyzed by real-time PCR for RV sequences, as shown in Fig. 1A. This demonstrated that liver contained 3.4 ± 1.25 RV DNA copies per 100 cells. This is consistent with the finding in our previous study that ~2% hepatocytes had high GUSB histochemical activity [32]. The DNA copies in spleen were 2-fold that in liver at 6.9 ± 1.6 copies per 100 cells. Bone marrow cells had 1.3 ± 0.43 copies per 100 cells, and there were 0.9 or fewer DNA copies per 100 cells in other organs. The possible contribution of blood-derived cells to the copy number in other organs is discussed below. Organs from a non-transduced dog whose DNA was isolated and amplified at the same time as the RV-transduced dogs did not have any DNA copies (data not shown), demonstrating a lack of contamination of samples or PCR reagents with RV sequences.

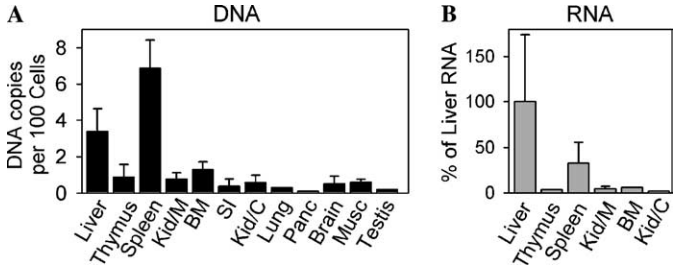

Fig. 1. Evaluation of DNA and RNA levels in organs of RV-treated dogs. RV-treated MPS VII dogs were injected with 3×10^9 TU/kg at 2–3 days after birth [29,32]. (A) DNA levels. For most organs, DNA was isolated from organs that were collected from M1312 and M1339 at 6–7 months after birth. For liver, DNA was isolated from biopsy samples obtained at 4 months after transduction from all five RV-treated dogs (M1312, M1339, M1328, M1332, and M1337). Real-time PCR was used to determine the RV DNA copy number, with normalization to the β-actin sequence. The average copies of RV per 100 cells ± SEM is shown. No RV DNA signal was observed for DNA isolated from organs of a non-transduced MPS VII dog (not shown). For testis, DNA was analyzed for only one animal (M1312). (B) RNA levels. Real-time reverse transcriptase (RT) PCR was performed with RNA from the RV-treated dogs. The percentage of RNA was normalized to the levels found in liver at 4 months for the RV-treated animals, which was defined as 100%. Spleen and kidney medulla were evaluated from both RV-treated animals, thymus was evaluated from M1312, and BM and kidney cortex were evaluated from M1339. Organs for which the RNA was degraded for both animals are not shown.

Expression of RV RNA in organs

Although the internal hAAT promoter was responsible for directing most of the expression from a similar RV in the liver at late times after transduction in rats [35], the LTR of the RV can also direct expression of an RNA that is translated into protein. Since expression in non-hepatic cells could contribute to correction of disease, other organs were evaluated for RV RNA transcripts by real-time PCR after RT treatment, as shown in Fig. 1B. Since the RNA obtained from the liver of M1312 and M1339 at post-mortum was degraded, liver RNA levels were evaluated in biopsy samples obtained at 4 months after RV transduction, and defined as 100%. Although, RNA levels could not be evaluated in some organs due to degradation of the RNA, RV RNA was detected in thymus (3% of liver after normalization to the β-actin signal) spleen (33 ± 23% of liver, $N = 2$), kidney medulla (4 ± 3% of liver, $N = 2$), bone marrow (6% of liver, $N = 1$), and kidney cortex (2% of liver; $N = 1$). There was no signal for the WPRE for RNA from a non-transduced liver that underwent real-time RT-PCR, and there was no signal for either the WPRE or β-actin if no RT was added to RNA from a transduced dog (data not shown). Thus, expression from the RV was substantial in spleen, and was detectable but low in the other organs that were evaluated.

Evaluation of blood for transduction and expression

Blood-derived cells could be the source of DNA and RNA sequences in other organs in dogs, as hematopoietic cells were transduced in mice with a similar neonatal gene therapy approach [33], and blood cells can migrate into other organs. Therefore, peripheral WBCs were evaluated for RV DNA and RNA sequences, and for GUSB activity. Figs. 2A and B show the result of a competitive PCR with analysis by gel electrophoresis. With this assay, the band derived from amplification of genomic DNA is longer due to the presence of an intron than the band derived from amplification of the cDNA in the RV. M1287, the HGF/RV-treated dog had ~1.6 copies per 100 cells at 0.3–4.3 years after transduction. Most of the RV-treated dogs had similar copies of RV at ~1.6 copies per 100 cells at 0.2 years and at a later time (0.3 years for M1312 and 4.1 years for M1328, M1332, and M1337). M1339 had fewer copies of DNA than the other RV-transduced dogs at ~0.4 copies per 100 cells at 0.2 years, and did not have a detectable signal with this assay at 0.3 years. However, there was a signal for M1339 at 0.3 years with a more sensitive PCR assay that recognizes the WPRE (data not shown). DNA from peripheral WBC from a non-transduced dog did not have

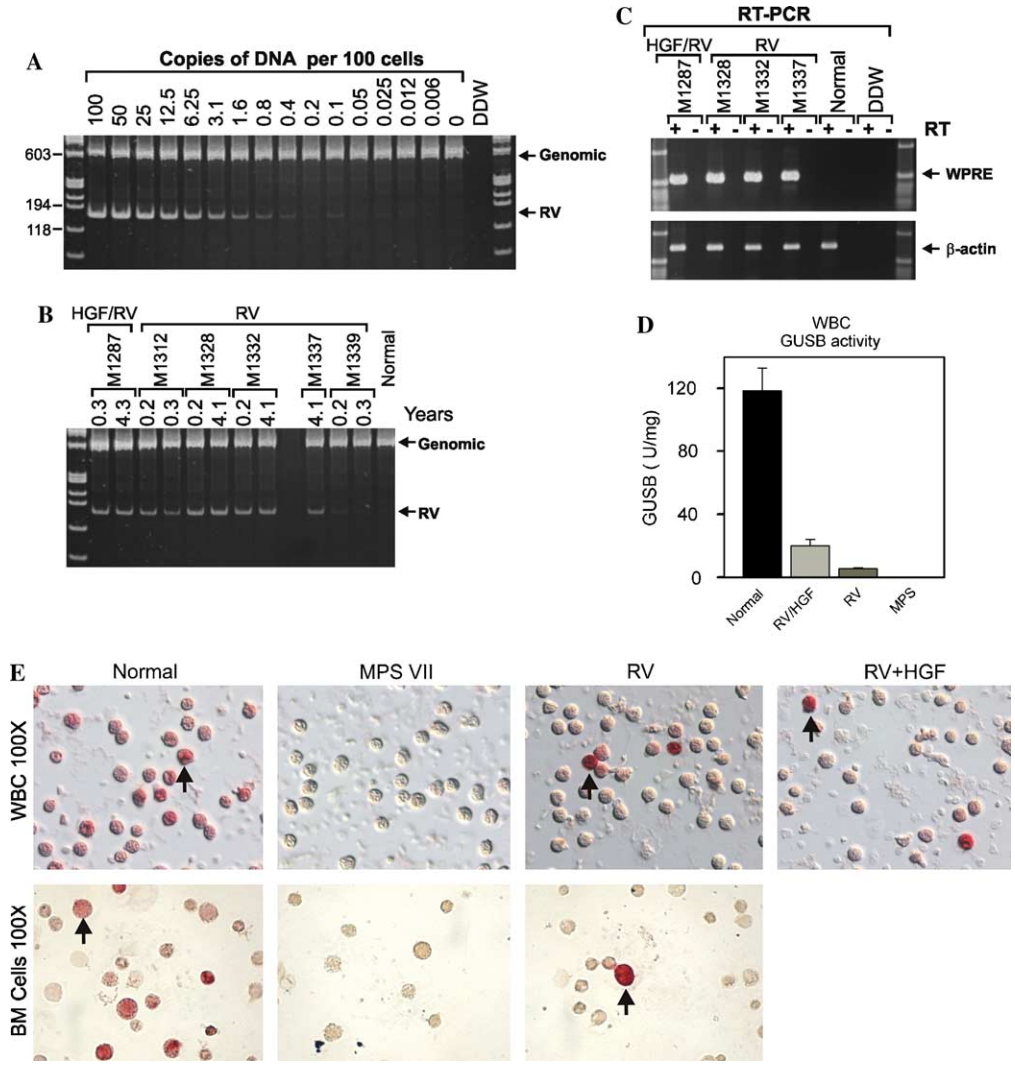

Fig. 2. Analysis of peripheral WBCs. (A) Standard curve for DNA. Competitive PCR was performed using DNA standards with the indicated copies of RV per 100 cells and primers from exons 4 and 5 of the cGUSB gene. The 621 bp band derived from genomic DNA (Genomic) and the 163 bp band derived from the cDNA of the RV are indicated at the right. The sizes in nt of DNA standards are indicated at the left. Samples that contained water without DNA (DDW) or DNA from a non-transduced dog (0) are shown. (B) RV DNA in treated MPS VII dogs. RV-treated dogs (RV) were transduced shortly after birth as described in Fig. 1. The HGF/RV-treated dog M1287 (HGF/RV) was treated with HGF at 2 days after birth and injected with 1.2×10^{10} TU/kg of hAAT-cGUSB-WPRE at 3 days after birth as described [29]. DNA was isolated from peripheral WBCs at the indicated years after transduction, and PCR was performed at the same time as the standard curve shown in panel A. The early sample of M1337 was lost, and normal represents PCR of DNA from a non-transduced dog. (C) RNA levels. RNA isolated from peripheral WBCs obtained at 2.8–2.6 years after HGF/RV- or RV-transduction, respectively, was treated with (+) or without (−) RT. PCR was performed using primers specific for the RV RNA (WPRE) or for β-actin sequences, as described in Materials and methods. RT-PCR on RNA from a non-transduced dog (normal) or for a sample with water only (DDW) is shown. (D) GUSB activity. GUSB activity in U/mg was determined in normal peripheral WBC ($N = 3$), peripheral WBCs collected at 2.9 and 4.4 years after HGF/RV transduction (M1287) or at 2.8 and 4.2 years after RV-transduction (M1328, M1332, and M1337), or peripheral WBCs from untreated MPS VII dogs ($N = 3$). The average GUSB activity ± SEM was determined for the two measurements for M1297, and the average of the average values was determined for the three RV-treated dogs. (E) GUSB histochemical activity in peripheral WBC and in BM cells. Peripheral WBCs were obtained from normal, untreated MPS VII, or RV-treated or HGF/RV-treated dogs at 4.2 or 4.4 years after transduction, respectively. BM cells were obtained from normal, untreated MPS VII, or from an RV-treated dog at 6 months after transduction. The black arrows indicate WBC with GUSB histochemical activity (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

any signal, demonstrating a lack of contamination. Similar results were obtained with peripheral WBC obtained at other times after transduction, as summarized in Table 2. DNA from peripheral WBC was also evaluated by real-time PCR at two times to quantify the RV copy number. The DNA copy number in blood cells from the HGF/RV-treated dog (M1287) at 2.8 and 4.3 years after transduction was 0.9 and 0.5 copies per 100 cells, respectively (Table 1). Similarly, DNA obtained from peripheral WBCs of RV-treated dogs at 2.6 and 4.1 years had 1.6 ± 0.6 and 1 ± 0.4 copies per 100 cells, respectively. No signal was obtained from a non-transduced dog, demonstrating the specificity of the reaction. Together, these data demonstrate that HGF/RV- and RV-treated dogs have ~1 copy of RV per

Table 1
Evaluation of peripheral WBCs for RV DNA and RNA levels

Neonatal RV transduction without HGF[a]		Non-transduced	Years after transduction					
			0.2	0.3	0.8	1.8	2.6	4.1
DNA	Competitive PCR (copies/100 cells)[b]	0	~1	~1	~1	~1	~1	~1
	Real-time PCR (copies/100 cells)[c]	0	NE	NE	NE	NE	1.6 ± 0.6	1 ± 0.4
RNA	Gel RT-PCR[d]	0	NE	NE	NE	NE	+	NE
	Real-time RT-PCR (percent of liver)[e]	0	NE	NE	NE	NE	NE	2.1 ± 0.8%
Neonatal RV transduction with HGF (M1297)[f]			0.3	0.5	1	2	2.8	4.3
DNA	Competitive PCR (copies/100 cells)[b]	0	~1	~1	~1	~1	~1	~1
	Real-time PCR (copies/ 100 cells)[c]	0	NE	NE	NE	NE	0.9	0.5
RNA	Gel RT-PCR[d]	0	NE	NE	NE	NE	+	NE
	Real-time RT-PCR (percent of liver)[e]	0	NE	NE	NE	NE	NE	1.1%

[a] Peripheral WBCs were obtained from RV-treated dogs at the indicated years after transduction. Samples were from M1328, M1332, and M1337 at 0.8 years or later, and from all five RV-treated dogs (those noted above, as well as M1312 and M1339) for the early times.
[b] Competitive PCR. Peripheral WBC DNA was analyzed by the competitive PCR followed by gel electrophoresis, as shown in Fig. 2A. The approximate copy number per 100 cells is shown.
[c] Real-time PCR. Peripheral WBC DNA was evaluated with real-time PCR for RV DNA sequences with normalization to the β-actin signal. The average copy number per 100 cells ± SEM is shown. NE indicates not evaluated.
[d] Gel RT-PCR. RNA was treated with RT followed by PCR and gel electrophoresis. + indicates that a signal for the RV was obtained.
[e] Real-time RT-PCR. RNA was treated with RT followed by real-time PCR with normalization to the β-actin signal. The signal relative to that found in liver ± SEM is shown.
[f] Peripheral WBCs were evaluated from the HGF/RV-treated dog M1297 at the indicated time after transduction.

100 cells, which appeared at 0.2 years and was maintained for over 4 years after transduction.

Since peripheral WBC had substantial copies of RV DNA, RNA from blood cells was evaluated to determine if the RV was expressed. RT-PCR followed by gel electrophoresis (Fig. 2C and Table 1) demonstrated that peripheral WBCs had detectable RV RNA at ~2.5 years after transduction for HGF/RV- and RV-treated dogs. No signal was observed without RT treatment, demonstrating that the signal was not due to contaminating DNA. A non-transduced dog had no signal for the RV after RT-PCR, although it was positive for β-actin. To further define the level of RNA, real-time PCR was performed after RT treatment, with normalization of the WPRE signal to the β-actin signal. This demonstrated that the RV RNA levels in peripheral WBCs at ~4 years after transduction of HGF/RV- or RV-treated dogs were 1.1 and 2.1 ± 0.8%, respectively, of that found in liver at 4 months after RV transduction. Use of RNA from a non-transduced dog, or RNA from a transduced dog that did not receive RT treatment, did not result in a signal, demonstrating the specificity of the reaction and the requirement for RNA, respectively. These data demonstrate that the RV is expressed in peripheral WBCs. Since, peripheral WBCs have 38% as many copies of RV per 100 cells as liver, this suggests that expression per copy is ~6% of that in liver cells.

Peripheral WBCs were also tested to determine if they contained GUSB activity. Fig. 2D demonstrates that the average GUSB activity in peripheral WBCs in the HGF/RV-treated dog was 20 ± 4 U/mg, which was 3.6-fold the value in the RV-treated dogs of 5.5 ± 0.4 U/mg. Since the relative peripheral WBC enzyme activity reflects the RV DNA copy number (1:1 for HGF/RV-treated:RV-treated), better than the ratio of serum activity (92:1 for HGF/RV-treated:RV-treated), it is likely that much of the activity in peripheral WBCs was derived from expression in transduced cells rather than uptake of enzyme from blood via the M6PR. The GUSB activity in peripheral WBC of RV-treated dogs was 28-fold that found in untreated MPS VII dogs (0.2 ± 0.01 U/mg) and was 5% of the value from cells of homozygous normal dogs (118 ± 15 U/mg).

Peripheral WBCs and BM cells were also stained for GUSB activity. Approximately half of the peripheral WBCs and BM cells from a normal dog stain red for GUSB activity, while none of the cells were positive from a non-transduced MPS VII dog (Fig. 2E). For an RV-treated dog, ~1% of the peripheral WBC and BM cells obtained at 4.5 years and 6 months after transduction, respectively, stained bright red, which is consistent with the DNA data showing ~1 copy of RV per 100 cells. Similarly, ~1% of the peripheral WBC of the HGF/RV-treated dog had high GUSB activity, although BM was not evaluated. The RNA and enzyme activity data suggest that BM and peripheral WBC produce GUSB, although the exact contribution of de novo expression vs. uptake of enzyme from blood is impossible to determine.

Effect of gene therapy on biochemical and pathological disease manifestations

We previously evaluated organ GUSB activity in two RV-treated dogs that were sacrificed at 6–7 months after birth [29]. The liver had the highest GUSB enzyme activity at 124 ± 66 (SEM) U/mg (30% normal). Activity in other organs was >1% normal, which is a level that would be expected to have a therapeutic effect. However, the effect

Table 2
Summary of histochemical analysis of GUSB enzyme activity and histological evaluation in organs

Organ	Cell type	GUSB activity			Lysosomal storage		
		Normal	MPS VII	RV	Normal	MPS VII	RV
Liver	Hepatocytes	+++	0	+++	0	+++	0
	Kupffer	+++	0	+++	0	+++	0
Thymus	Cortex	+++	0	++	0	++	0,+
	Medulla	+++	0	++	0	+++	0
Spleen	Red pulp	+++	0	+++	0	++, +++	0
	Germinal center	+	0	+	0	+	0
Kidney	Tubules	+++	0	+/−	0	+++	+
	Glomeruli	++	0	++	0	+	0
	Interstitium	++	0	+/−	0	+++	0
Small intestine	Lamina propria	+++	0	++	0	++	0
	Submucosa	+++	0	+	0	+++	0
	Muscularis externa	+	0	0	0	++	0
Lung	Parenchyma	+++	0	0	0	+++	0
	Bronchi	+++	0	NE	0	++	NE
Pancreas	Acini	+++	0	0	0	0	0
	Interstitium	+++	0	0	0	++	0
	Islets	+	0	0	0	0	0
Brain	Hippocampus neurons	NE	NE	NE	0	++, +++, +++	0,+
	Cortex neurons	+/−	0	0	0	++, +++, +++	0, +
	Purkinje cells	NE	NE	NE	0	++, +++, +++	0, ++
	Astrocytes	NE	NE	NE	0	+++	0,+
	Perivascular	0	0	0	0	+++	0, +
	Meninges	++	0	+/−	0	+++	0
Eye	Cornea	NE	NE	NE	0	+++	+
Muscle	Myocytes	0	0	0	0	0	0
	Interstitium	+/−	0	0	0	++, +++	0
Testis	Tubules	NE	NE	NE	0	0	0
	Interstitium	NE	NE	NE	0	0	0

Normal, untreated MPS VII, and RV-treated (M1312 and M1339) dogs were treated as described in Fig. 1. They had serum GUSB activity of 269 ± 17 U/ml, 0.55 ± 0.05 U/ml, and 149 ± 17 U/ml, respectively. Animals were sacrificed at 6 to 7 months after birth. Frozen sections from one normal, one untreated MPS VII, and both RV-treated dogs were stained for GUSB activity, as shown in Figs. 5 and 6. GUSB activity was scored as 0 (no detectable red color), +/− (probable weak activity in some cells), + (definite low activity in some cells), ++ (moderate activity in many cells), or +++ (activity in all cells). Thin sections from fixed tissue of one normal, two (occasionally three) untreated MPS VII, and both RV-treated dogs were stained with toluidine blue to evaluate for lysosomal storage. Samples from three different untreated MPS VII dogs were evaluated for lysosomal storage in the brain. Lysosomal storage was scored as 0 (none detected), + (low amounts of storage in some cells), ++ (moderate storage in many cells), or +++ (severe storage in most cells). If all animals were concordant, one value is shown. If values for the group were discordant, values for each animal evaluated are shown. NE indicates not evaluated.

upon biochemical and pathological manifestations of lysosomal storage has not yet been evaluated. We therefore tested levels of GAG and β-Hex activity in RV-treated dogs.

Optimization of a sulfated GAG assay

Determination of GAG levels in organs is important to determine the degree of correction of lysosomal storage. A simple and commercially available assay was tested, in which sulfated GAGs precipitate a dye, which can be quantified by measuring the OD at 655 nm after dissolving the pellet. An initial experiment tested if high levels of protein in the samples would affect the precipitation of dye by chondroitin sulfate (CS) standards. Fig. 3 demonstrates that the signal for CS standards was not affected by the addition of 150 μg of protein from the extracts of normal liver or spleen as long as the OD was below 1.0. However, addition of higher amounts of protein from the extracts resulted in a reduction of the signal for the standards, suggesting that it was inhibiting precipitation of the dye. Therefore, assays were performed with 150 μg or less of extract protein, and results were only accepted if the OD was less than 1.

GAG levels in organs

Soluble sulfated GAG levels increase in organs from animals with MPS VII, and normalization of GAGs correlates well with reduction in lysosomal storage. As shown in Fig. 4A, soluble sulfated GAGs were significantly increased at 6–7 months of age in untreated MPS VII dogs as compared with values in normal dogs in thymus (15 ± 3 μg GAG/mg protein; 20-fold normal), spleen (13.5 ± 5 μg GAG/mg protein; 20-fold normal), kidney medulla (19 ± 8 μg GAG/mg protein; 48-fold normal), BM (18 ± 5 μg GAG/mg protein; 29-fold normal) small intestine (16 ± 6 μg GAG/mg protein; 9-fold normal), kidney cortex (12 ± 3 μg GAG/mg protein; 45-fold normal), lung (16 ± 3 μg GAG/mg protein; 34-fold normal), and pancreas (5 ± 2 μg GAG/mg protein; 5-fold normal).

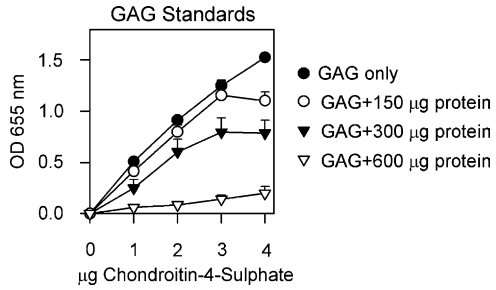

Fig. 3. Effect of protein in extract upon the GAG assay. The GAG assay was performed with 0–4 μg of CS standard as described in Materials and methods. One set of standards did not contain any extract from organs (GAG only), while other sets of standards had 150, 300, or 600 μg of protein from organ extracts in addition to the CS standards. For the standards with extract protein added, two extracts were from normal dog liver and one was from normal dog spleen. For all sets, the background OD obtained at 595 nm for samples that did not receive any CS was subtracted from the other values, and the average OD above this background ± SEM for three assays is shown.

Although soluble GAGs were 3-fold normal at 3 ± 2 μg GAG/mg protein in liver from untreated MPS VII dogs, these values were not statistically different from those in normal dogs. There was no elevation in GAG levels in the parietal cortex of brain (5 ± 1 μg GAG/mg protein) or muscle (0.3 ± 0.2 μg GAG/mg protein) of untreated MPS VII dogs when compared with values in normal dogs, suggesting that evaluation of GAGs in these organs will not allow a therapeutic response to be evaluated. RV-treated MPS VII dogs had a significant reduction in GAG levels to normal in liver, thymus, spleen, and pancreas when values were compared with those in untreated MPS VII dogs. Values in RV-treated dogs were also statistically lower than in untreated MPS VII dogs in kidney medulla (RV-treated had 4% of untreated MPS VII GAG levels), BM (8% of MPS VII), kidney cortex (9% of MPS VII), and lung (6% of MPS VII), although the levels were 2- to 4-fold the values in normal dogs. These data suggest that reductions in GAGs may be only partial in kidney, BM, and lung, although the values in these organs in RV-treated dogs were not statistically different from those in normal dogs due to the small number of samples analyzed. GAG levels in the small intestine of RV-treated dogs were reduced to 3-fold normal, although this was not statistically different from the values in untreated MPS VII dogs.

β-Hex activity in organs

Mucopolysaccharidosis VII results in an elevation in the activity of other lysosomal enzymes, which may reflect an increased total mass of lysosomes, or alterations in gene expression. Since normalization of this elevation of second-

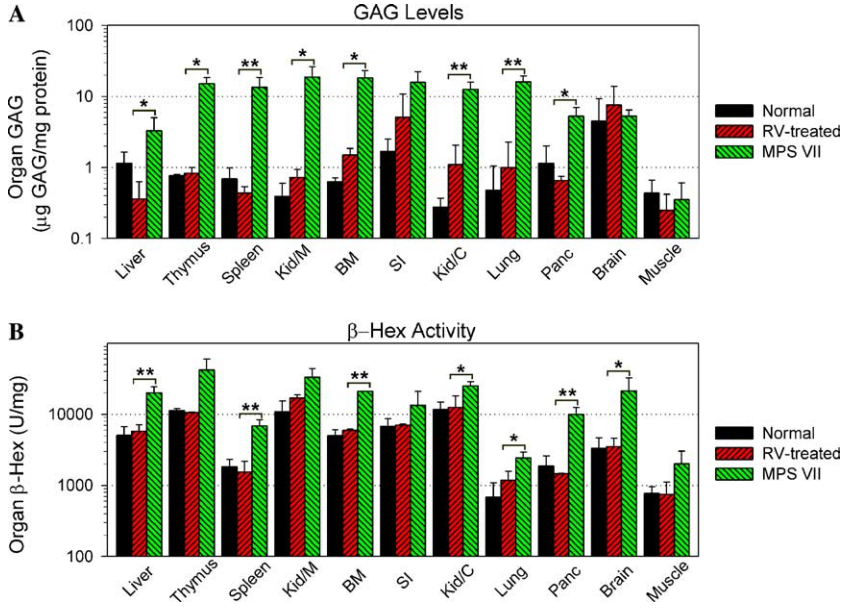

Fig. 4. Analysis of organ extracts for lysosomal enzyme and GAG levels. Dogs were normal, RV-treated MPS VII animals that were injected with RV at 2 or 3 days after birth as described in Fig. 1 (M1312 and M1339), or untreated MPS VII animals (MPS VII). Dogs were sacrificed at 6–7 months after birth and homogenates prepared from organs. For most organs, samples were collected from 4 to 5 normal, both RV-treated MPS VII, and 3 to 4 untreated MPS VII dogs. Only two samples were evaluated from the thymus of normal dogs. The same samples were tested for GAG levels and β-Hex activity. (A) GAG levels. Semi-log plot of the average sulfated microgram GAG per milligram protein ±SD in organs of homozygous normal, RV-treated, and untreated MPS VII dogs ±SEM are shown. Results in liver appear at the left, and results in other organs are organized from left (highest activity) to right (lowest activity) according to the GUSB activity in U/mg in the RV-treated dogs. Abbreviations are kidney medulla (Kid/M), bone marrow (BM), small intestine (SI), kidney cortex (Kid/C), and pancreas (Panc). Values that were significantly different using ANOVA between normal and RV-treated dogs, or between RV-treated MPS VII and untreated MPS VII dogs, are indicated as an asterisk above a bar connecting the two groups. $^{*}p = 0.01$–0.05 and $^{**}p < 0.01$. (B) β-Hex activity. The average levels of the secondary lysosomal enzyme β-Hex ± SD was determined for the same samples described in (A), and statistical comparisons performed as in (A).

ary lysosomal enzyme activities by effective treatment correlates well with improvements in lysosomal storage, organs were tested for β-Hex activity, as shown in Fig. 4B. β-Hex activity in extracts from untreated MPS VII dogs were 2- to 6-fold that in normal animals in all organs that were evaluated. This difference was significant ($p < 0.05$ with ANOVA for MPS VII vs. normal) for all organs except thymus, small intestine, and muscle. Neonatal RV-mediated gene therapy resulted in significant reductions in the β-Hex activity in liver, spleen, BM, kidney cortex, lung, pancreas, and the parietal cortex of brain ($p < 0.05$ for comparison of RV-treated vs. untreated MPS VII samples). Normalization of β-Hex levels in brain is of particular interest, as brain is an important site of disease, and improvements in brain have been difficult to achieve with some approaches in mice. β-Hex activity was also reduced in RV-treated MPS VII dogs in thymus, kidney medulla, small intestine, and muscle. However, values in these organs in RV-treated MPS VII dogs were not statistically lower than values in untreated MPS VII dogs, which may be due to the small number of samples analyzed. There were no significant differences in β-Hex activity in any organ of RV-treated dogs when comparisons were made to samples from normal dogs. The GAG and β-Hex data suggest that RV-treated dogs had substantial correction in lysosomal storage throughout the body.

Histochemical analysis for GUSB activity and pathological evaluation for lysosomal storage

Organs were evaluated with histochemistry for GUSB activity to determine the cells that contained enzyme, and with histopathology with TB staining of thin sections to determine if cells contained lysosomal storage. Table 2 summarizes the results of these studies, while representative examples of staining are shown in Figs. 5 and 6. RV-treated MPS VII dogs had high levels of histochemically visible GUSB activity in liver (Table 2), thymus (Fig. 5A) and spleen (Fig. 5C), which resulted in a marked reduction in histological evidence of lysosomal storage in these organs compared with untreated MPS VII dogs (Table 2, Figs. 5B and D). Although, RV-treated dogs had histochemically visible GUSB activity in the glomeruli of the kidney, little activity was visible in the tubules (Fig. 5E). Nevertheless, there was partial reduction in lysosomal storage in the tubules (Fig. 5F), which was consistent with the partial reduction in GAG levels. The tubules probably contained some GUSB activity, but the level was insufficient to result in visible red with the stain.

There was very little histochemically visible GUSB activity in the parietal cortex of the brain of RV-treated dogs, as shown in Fig. 6A. Nevertheless, lysosomal storage was markedly reduced in neurons in the hippocampus (Figs. 6B and C) and the brain cortex (Fig. 6D), or in Purkinje cells of the cerebellum (Fig. 6E) of one RV-treated dog (M1312). Storage was detected in neurons in the other RV-treated dog (M1339), but was clearly reduced compared with untreated MPS VII dogs, which had large amounts of lysosomal storage in most cells. Storage was also reduced in RV-treated MPS VII dogs in astrocytes, the perivascular region, and the meninges (Table 2). Reduction in storage in the cornea (Table 2) correlates with the improvement in corneal clouding on opthalmologic examination that was reported previously [29]. Thus, neonatal gene therapy reduced pathology in organs, including brain, that are typically affected by MPS VII.

Urine GAG levels

Urine GAGs are frequently elevated in patients with MPS, and effective treatment can reduce their levels. Urine GAGs were elevated in untreated MPS VII dogs at an average level of 89 ± 21 μg GAG/mg creatinine, which was statistically higher ($p < 0.01$ using ANOVA) than the average value of 6 ± 2 μg GAG/mg creatinine in normal dogs (Fig. 7). RV-treated dogs had normal urine GAG levels at 8 ± 1 μg GAG/mg creatinine, which was statistically lower than the values in untreated MPS VII dogs ($p < 0.01$).

Discussion

Spleen cells are transduced after neonatal IV injection of RV

Identification of the site of transduction and expression is important to understand the mechanism by which correction of MPS VII occurs after gene therapy. We had previously hypothesized that secretion of M6P-modified GUSB from liver cells into blood and subsequent uptake by cells in other organs was the major mechanism of correction in MPS VII dogs for the reasons stated in the introduction. However, the data presented here suggest that expression in other organs might contribute to correction of disease. The spleen had 6.9 ± 1.1 copies of RV per 100 cells at 6 months, which is consistent with our previous result demonstrating efficient transduction of spleen at 1 week after neonatal transduction in dogs [32], and suggests that transduced splenocytes survive for that period of time. Transduction of spleen is not surprising, as the spleen contains replicating cells with access to the RV in the blood. It is unclear why copies in the spleen were twice that in liver (3.4 ± 0.6 RV DNA copies per 100 cells) at 6 months, as liver and spleen had similar copies of DNA at 1 week after transduction in a previous study [32]. Transduction of short-lived cells in the liver, such as Kupffer cells or hematopoietic progenitor cells, may have contributed to the higher relative copy number in the liver at the earlier time.

Expression occurred in the spleen, as RV RNA levels were $33 \pm 16\%$ of that in liver after normalization to the β-actin signal. Since the spleen had more copies of DNA than the liver, this suggests that the expression per copy of RV in spleen was only 16% of that in liver. Nevertheless, expression was substantial, and likely contributed to correction of disease in spleen. It is also possible that splenocytes secreted GUSB into blood, which played a role in correction of disease in other organs. Transcription in

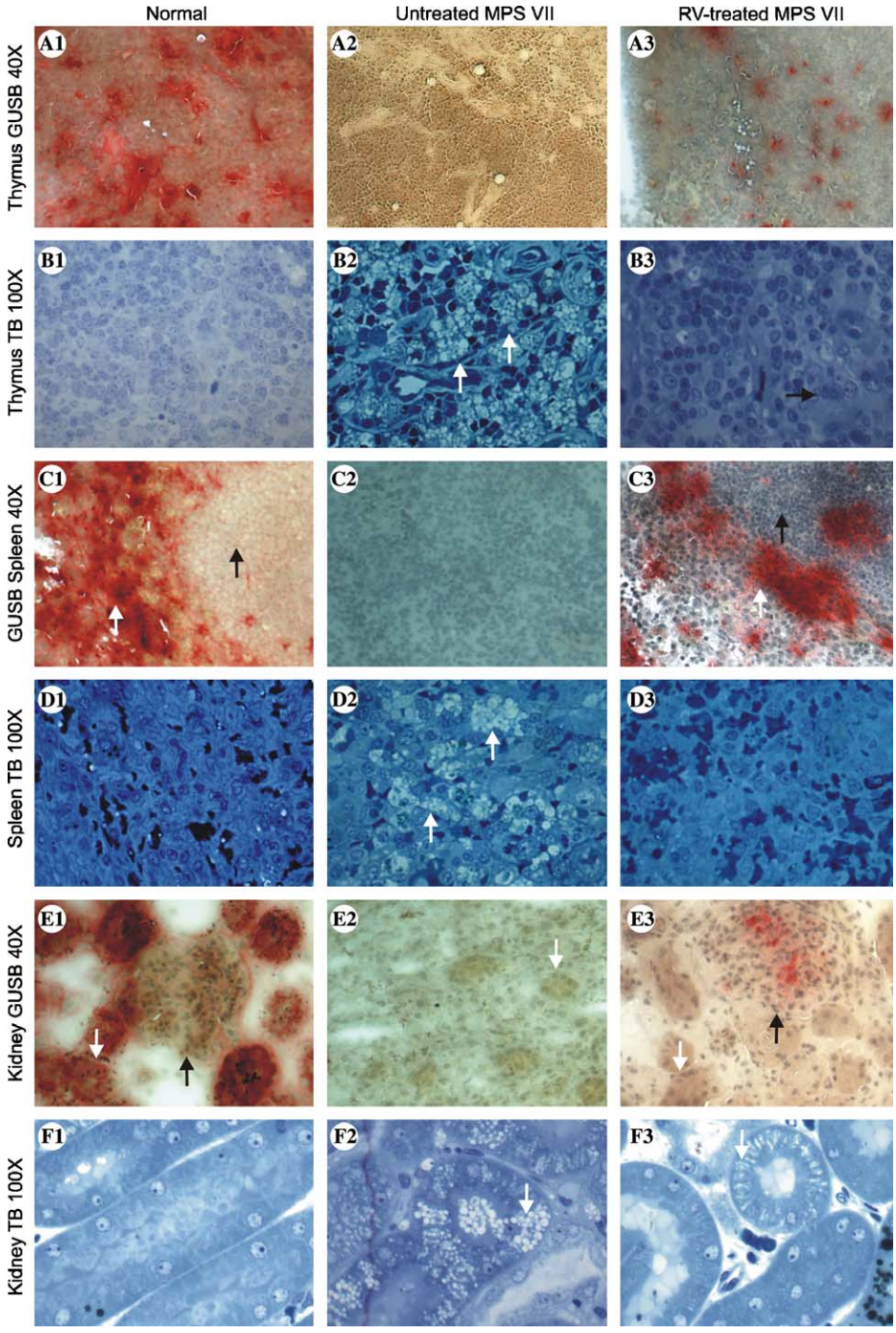

Fig. 5. Histochemical and histopathological analysis of thymus, spleen, and kidney. Dogs were treated as described in Fig. 1, and organs were collected at 6–7 months after birth. For panels indicated with GUSB at the left, frozen sections were stained overnight for GUSB activity (red in the cytoplasm) and counterstained with hematoxylin (blue in the nucleus). For panels indicated with TB at the left, thin sections of fixed organs were stained with toluidine blue, which does not stain lysosomes. The original magnification is shown at the left. Representative examples from normal (column 1, labeled with a 1), untreated MPS VII (column 2, labeled with a 2), and RV-treated MPS VII (column 3, labeled with a 3) are shown. M1312 and M1339 had identical scores for the amount of lysosomal storage from the regions of organs shown in this figure. (A) GUSB stain of thymus. Normal and RV-treated dogs have substantial GUSB activity in both the cortex and the medulla, but there is no activity in untreated MPS VII dogs. (B) TB of thymus medulla. The untreated MPS VII dog has a substantial amount of lysosomal storage in the medulla (white arrows), which is absent in the RV-treated MPS VII dog. (C) GUSB of spleen. Normal and RV-treated dogs have substantial GUSB activity throughout the red pulp (white arrow), and little activity in the white pulp (black arrow). (D) TB of spleen. Untreated MPS VII dogs have substantial amounts of storage in the red pulp (white arrow), which is absent in RV-treated dogs. (E) GUSB of kidney cortex. Normal dogs have very high activity in the tubules (white arrow) and less activity in the glomeruli (black arrow). RV-treated dogs have high enzyme activity in the glomeruli, but little activity in the tubules. (F) TB stain of kidney cortex. The tubules from an untreated MPS VII dog have a large amount of lysosomal storage (white arrow). Storage is present at reduced levels in an RV-treated MPS VII dog. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

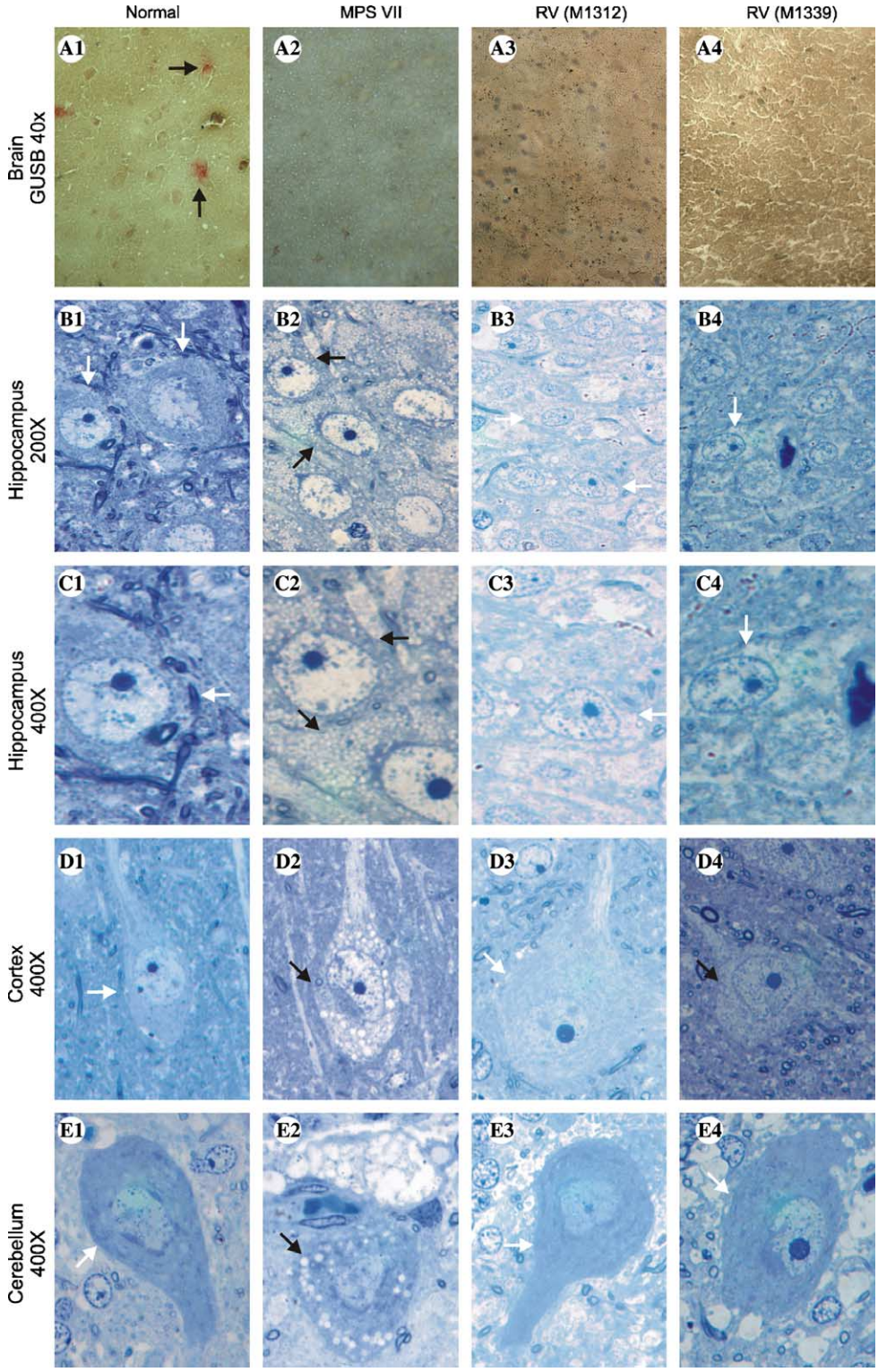

Fig. 6. Histochemical and histopathological analysis of brain. Animals and samples were treated as described in Fig. 1, and panels are labeled as noted in Fig. 5 except examples of staining are shown for both of the RV-treated dogs (M1312 in column 3 and M1339 in column 4). (A) GUSB stain of parietal cortex of brain. The normal dog has a small amount of GUSB activity (black arrows) in the parietal brain cortex. No GUSB staining is apparent in untreated or RV-treated MPS VII dogs. (B and C) TB stain of hippocampus. The untreated MPS VII dog has substantial amounts of storage in neurons of the hippocampus (black arrows). White arrows identify neurons without storage. (D) TB stain of parietal cortex. Lysosomal storage in a neuron (black arrow) is substantial in an untreated MPS VII dog, and present in small amounts in M1339. White arrows identify neurons without lysosomal storage. (E) TB stain of cerebellum. The untreated MPS VII dog has substantial amounts of storage in a Purkinje cell of the cerebellum (black arrow). White arrows identify Purkinje cells without storage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

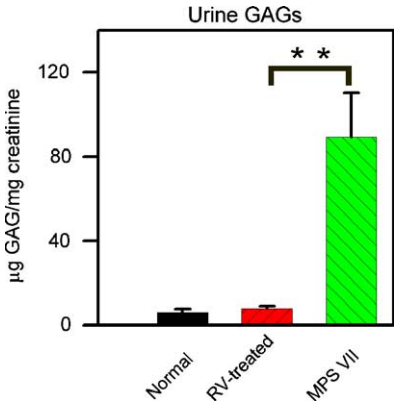

Fig. 7. Urine GAG. Urine was collected at 6 months to 1.5 years after birth from normal dogs ($N = 16$), at 6 months to 3 years after neonatal RV transduction ($N = 9$), or at 6 months to 2.4 years after birth for untreated MPS VII dogs ($N = 10$). Urine GAGs were normalized to the creatinine in the same sample and plotted as the average ± SD. Values in RV-treated dogs were statistically lower than in untreated MPS VII dogs using ANOVA, with a $^{**}p < 0.01$. Values in RV-treated dogs were not statistically different from values in normal dogs.

spleen most likely initiated from the LTR, which can drive expression of an RNA that is translated into protein in a variety of cell types. Although the LTR of an LNL6-based vector (as was used here) can shut-off over time in mice, less repression occurs in human-derived cells [36] and canine-derived cells may also be less prone to shut-off. These data in dogs differ from those in mice that received a similar neonatal gene therapy approach, where the copies of RV DNA and RNA in spleen were 10 and 1%, respectively, of that in liver at 6 months after transduction [26].

Hematopoietic cells are transduced after neonatal IV injection of RV

The demonstration here that organs other than liver or spleen had DNA copies that were 3–26% of that in liver at 6 months was surprising, as DNA copies in organs other than liver or spleen were <1% of that in liver at 1 week after transduction in a previous study in dogs [32]. There were also low levels (2–6% of the level in liver) of RV RNA in the organs that could be evaluated (thymus, kidney, and BM), suggesting that expression in other organs could contribute to correction of disease. Since both BM and peripheral WBC of RV-treated dogs had 1.3 copies of RV per 100 cells, and, respectively, had 6 and 2% as much RNA as did liver, it is possible that transduced hematopoietic cells that migrated over time into other organs, or remained in the intravascular space despite perfusion, may have contributed to the DNA and RNA copies in other organs. It is possible that cells in these other organs secreted GUSB into blood, although this contribution would likely be small as the relative RV RNA levels were low. The higher expression in non-hepatic organs of dogs than in mice may reflect higher RV DNA copies in other organs in dogs than in mice [26]. In addition, the LTR may be less likely to shut-off over time in large animals than in mice [36].

In this study, HSC were probably transduced after neonatal IV injection of RV, as DNA sequences remained at stable levels in peripheral WBCs for over 4 years after transduction. This could have occurred in the liver, which has a substantial amount of hematopoiesis at 2–3 days after birth (data not shown). Hematopoietic stem cells might also be transduced in blood, as cord blood has substantial numbers of these cells. Thus, a simple IV injection of RV might transduce HSC for gene therapy of blood diseases where a low percentage of modified cells would exert a clinical benefit.

Correction of disease in somatic organs

Neonatal IV injection of RV dramatically reduced the biochemical and pathological manifestations of lysosomal storage in MPS VII dogs in liver, thymus, spleen, BM, small intestine, and lung. There was also a substantial improvement in evidence of lysosomal storage in the tubules of the kidney cortex, although GAG levels remained partially elevated, and clear-cut pathological evidence of storage was present in the RV-treated dogs. This is consistent with the difficulty in reducing storage in kidney tubules with gene therapy in mice with MPS VII [23,26]. In addition, urine GAGs were reduced to normal levels.

Reduction in lysosomal storage in brain

An exciting result was the reduction in evidence of lysosomal storage in the brain. First, activity of a secondary lysosomal enzyme (β-Hex) was normalized in the brain cortex of RV-treated dogs. Second, there was complete and partial correction of pathological evidence of lysosomal storage in neurons in the RV-treated dogs M1312 and M1339, respectively, at 6 months after transduction. The effect of gene therapy on GAG levels in brain could not be evaluated, as GAGs were not elevated in untreated MPS VII as compared with normal dogs.

There are two potential mechanisms for improvement of disease of brain. First, the GUSB that is present at high levels in serum could enter the brain. In mice, the newborn brain took up GUSB from blood in a M6PR-dependent fashion after IV injection of enzyme [37]. In addition, ERT initiated at birth reduced lysosomal storage in neurons [15], while liver-directed neonatal gene therapy with AAV [22], RV [26], or adenoviral [24] vectors reduced storage in the brain. It is also possible that enzyme could enter the brain at a low rate after the neonatal period. Although it was reported that uptake of GUSB by brain was not detectable in adult mice [37], it is possible that uptake occurred at a level below the detection limit, and that the continuous presence of high levels of enzyme in blood could allow enzyme to reach brain at low levels. Indeed, adult MPS VII mice that received ERT had a modest reduction in pathology in cortical neurons [15], while IV injection of an AAV vector to adult MPS VII

mice resulted in a profound reduction in storage in brain [23].

The second possible mechanism of reduction of storage in the brain is that the transduced blood cells migrated into the brain and secreted enzyme that was taken up by adjacent cells. Indeed, HSCT at birth in MPS VII mice [9] or HSC-directed gene therapy in mice with other lysosomal storage diseases [38] reduced storage in neurons. In addition, HSCT within 2–3 years after birth in human patients has probably improved neurological outcomes [12]. It is intriguing that M1339, the RV-treated dog with the fewest copies of RV in peripheral WBCs, had more severe storage in brain than M1312, although further analyses will need to be performed to determine if correction in brain correlates with the transduction efficiency in hematopoietic cells. Although there are no formal data demonstrating an improvement in neurological function in RV-treated dogs in this study, one HGF/RV- and three RV-treated MPS VII dogs are currently over 4.5 years old, and function normally. However, it will be important to evaluate the brains of these RV-treated dogs at a late time after transduction to determine if pathological improvements are maintained.

Evaluation for germline transmission

One RV-treated male (M1312) had low copies of RV DNA in his testes at 0.2 copies per 100 cells at 6 months after transduction, and semen collected from the HGF/RV-treated male (M1287) and one RV-treated male (M1328) at various times from 1 to 4 years after transduction had low copies of RV sequences (data not shown). This raises the concern that germline transmission might have occurred. However, isolated sperm from these dogs was negative for RV DNA sequences (data not shown), which suggests that the DNA sequences in semen may have derived from contaminating WBC. The HGF/RV- and the RV-treated males have fathered 90 and 172 offspring, respectively, none of which have contained RV vector sequences in their blood cells (data not shown). These data suggest that the risk of germline transmission is low.

Implications for gene therapy

These data demonstrate that both secretion of GUSB into blood from liver and spleen, and expression in transduced hematopoietic cells, may contribute to the profound improvement in biochemical and pathological manifestations seen in MPS VII dogs after neonatal RV transduction. As it is currently impossible to determine the relative contributions of each of these mechanisms to the correction of disease, we are developing an RV that will have liver-restricted expression to address this issue. Regardless of the major mechanism of correction, neonatal IV injection of RV is a remarkably simple and effective treatment for dogs with MPS VII, and has not had any adverse effects in the four dogs that have been evaluated for 4.5 years to date. If safety concerns can be adequately addressed, this treatment could have a dramatic effect on the lives of patients with MPS and their families.

Acknowledgments

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