Correction of Clinical Manifestations of Canine Mucopolysaccharidosis I with Neonatal Retroviral Vector Gene Therapy

Anne M Traas[1,2], Ping Wang[1,2], Xiucui Ma[3], Mindy Tittiger[3], Laura Schaller[3], Patricia O'donnell[1,2], Meg M Sleeper[1,2], Charles Vite[1,2], Ramin Herati[3], Gustavo D Aguirre[1,2], Mark Haskins[1,2] and Katherine P Ponder[3]

[1]Department of Clinical Studies, University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania, USA; [2]Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania, USA; [3]Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA

Mucopolysaccharidosis I (MPS I) (Hurler syndrome) is due to deficient α-L-iduronidase (IDUA) activity and is the most common of the MPS disorders. Neonatal MPS I dogs were injected intravenously (IV) with a gamma retroviral vector containing a complete long-terminal repeat (LTR) and an internal human α_1-antitrypsin (hAAT) promoter upstream of the canine IDUA complementary DNA (cDNA). This resulted in stable serum IDUA activity of 366 ± 344 units (U)/ml (28-fold normal) for up to 1.8 years, which likely derived primarily from secretion of IDUA by transduced liver cells. Retroviral vector (RV)-treated dogs had >18% of normal IDUA activity in organs and had decreased severity and/or incidence of hernias, chest deformities, joint disease, facial dysmorphia, corneal clouding, valvular heart disease, and aortic dilatation as compared with untreated MPS I dogs. The marked reduction that was observed in lysosomal storage in the brain of RV-treated dogs may have been due in part to expression from the LTR of the vector in cells in the brain. This possibility will be explored in future studies, because the potential for insertional mutagenesis has raised concerns about using vectors with an intact LTR. If proven safe, this gene therapy technique may be utilized in treating children with Hurler syndrome.

Received 12 January 2007; accepted 7 April 2007; pulished online 22 May 2007. doi:10.1038/sj.mt.6300201

INTRODUCTION

Mucopolysaccharidosis I (MPS I) is a lysosomal storage disease caused by deficient α-l-iduronidase (IDUA) activity that leads to the accumulation of the glycosaminoglycans (GAG) dermatan and heparan sulfate. It is the most common MPS, affecting nearly 1 in 100,000 live births.[1] In the severe Hurler syndrome, patients have umbilical hernias, skeletal deformities, joint disease, facial dysmorphia, corneal clouding, aortic dilatation, valvular heart disease, mental retardation, chronic rhinitis, recurrent middle ear infections, and liver and spleen enlargement. The attenuated Scheie syndrome has reduced systemic symptoms without neurological involvement, whereas the Hurler–Scheie syndrome is intermediate in severity. The canine model of MPS I is caused by a G to A transition in the 5′ splice site of the first intron[2,3] and results in disease similar to that in humans. However, dogs also have thickening and prolapse of the third eyelid (membrana nictitans), as well as joint laxity instead of joint stiffness; they do not have obvious cognitive impairment, rhinitis, or middle ear infections.

MPS I is currently treated with hematopoetic stem cell transplant (HSCT) or enzyme replacement therapy (ERT). Lysosomal enzymes acquire mannose 6-phosphate (M6P) during transport through the Golgi, which allows the protein to bind to the M6P receptor and translocate to the lysosome. M6P-modified enzyme that is secreted by blood-derived cells or is injected intravenously (IV) can be taken up by cells via the M6P receptor. HSCT can reduce some clinical signs of disease, although bone, cartilage, cardiac, neurologic, and corneal manifestations were not completely corrected.[4–9] Major disadvantages of HSCT include the need for a compatible donor, the expense, and the ~15% mortality rate. ERT has reduced hepatosplenomegaly, improved growth when given before puberty, increased mobility and manual dexterity, and reduced pulmonary and cardiac symptoms, although aortic insufficiency progressed with time.[10–13] The high cost of ERT, more than US$500,000 per year for a 50 kg patient, is a major drawback for this long-term treatment.

Systemic gene therapy could program some cells to secrete IDUA, which could be taken up by other cells via the M6P receptor. Indeed, neonatal gene therapy in MPS I mice with a gamma retroviral vector (RV),[14,15] a lentiviral vector,[16] or an adeno-associated virus vector[17] resulted in stable IDUA expression and reduced clinical manifestations. Extending these studies to a large animal model such as the MPS I dog is important, as their response will probably be more predictive of results in humans. However, previous attempts at gene therapy in the MPS I dog failed to induce long-term expression of IDUA owing to

Correspondence: *Katherine P. Ponder, 660 S. Euclid Avenue,Washington University School of Medicine, Box 8125, St. Louis, Missouri 63110, USA. E-mail: kponder@im.wustl.edu*

an immune response, failure of cell engraftment, or low transduction efficiency.[18–22] We recently reported that neonatal IV injection of a canine IDUA-expressing gamma RV resulted in stable serum IDUA activity in MPS I dogs for up to 1.5 years without an immune response.[23] We report herein that this results in marked reductions in clinical, biochemical, and pathological manifestations of disease.

RESULTS

Neonatal gene therapy results in stable serum IDUA activity

Nine MPS I dogs were injected IV at 2–3 days of age with an average dose of $4.3 \pm 3.4 \times 10^9$ transducing units/kg of hAAT-cIDUA-WPRE, as summarized in **Table 1**. This RV has intact long-terminal repeats (LTRs) but can also express canine IDUA from an internal liver-specific human α_1-antitrypsin promoter.[23] All six RV-treated dogs that survived long term had stable serum IDUA activity for 1 year or longer that averaged 366 ± 344 units (U)/ml.[23] Two animals (I-99 and I-101) had relatively low serum IDUA activity at 23 and 27 U/ml, respectively (twofold normal) and will be referred to hereafter as RV-treated dogs with low serum IDUA activity. Four animals (I-107, I-140, I-171, and I-172) had average serum IDUA activity of 553, 888, 240, and 608 U/ml, respectively (overall average 535 ± 285 U/ml, 41-fold normal) and will be referred to hereafter as RV-treated dogs with high serum IDUA activity. The IDUA activity in individual dogs did not correlate well with the RV dose. Untreated MPS I dogs had very low serum IDUA activity at 0.5 ± 0.1 U/ml.

Survival of RV-treated dogs

Two of nine RV-treated dogs (22%) died before 2 weeks of age. Post-mortem evaluation did not find evidence of inflammation, and the cause of these early deaths was not clear. This frequency was not significantly different from the 20% death rate in 10 untreated MPS I puppies between 2 days and 2 weeks after birth. In addition, 17% of untreated MPS I puppies that were alive at birth died within 2 days ($N = 23$), further suggesting that early deaths may be due to the poor viability of MPS I puppies rather than the gene therapy. Of the remaining seven RV-treated dogs, one died at 2.5 months of an unclear etiology at post-mortem and another developed a mild gait problem owing to luxation of both shoulders at 12 months and was euthanized to obtain tissue for analysis; the remaining five RV-treated dogs were alive and well at the last evaluation (at 18 months) for two dogs or at the time of euthanasia (at 21, 19, and 15 months). All untreated MPS I dogs that were alive at 2 weeks survived at least 6 months. However, of four untreated MPS I dogs that were followed for 12–21 months, two dogs (50%) developed neurological disease and were euthanized, as will be discussed further below.

Clinical improvement in RV-treated MPS I dogs

The RV-treated animals showed improvements in many of the clinical signs associated with MPS I, as summarized in **Table 1**. For example, all untreated MPS I dogs had umbilical hernias, but hernias were absent or had closed over time in all RV-treated dogs. Two of five untreated MPS I dogs had chest deformities characterized by a sigmoid curve of the ventral aspect of the caudal ribcage, resulting in mild flattening of the chest. Chest deformities were absent in all RV-treated MPS I dogs. Joint disease was noted in four of five untreated MPS I dogs and involved effusion, laxity, crepitus on manipulation, and/or deformity of the normal joint angle of the elbows, carpi, phalanges, and/or stifles (knees). No RV-treated dogs had clinical signs of joint disease. RV-treated dogs also showed decreased skeletal abnormalities, as will be discussed in a future publication.

Facial appearance

Untreated MPS I dogs had a characteristic depressed bridge of the nose; this resulted in a curved muzzle that was apparent at 3–6 months of age and remained abnormal thereafter, as illustrated at 1 year in **Figure 1b**. In contrast to MPS VII dogs, the snout length of MPS I dogs appeared relatively normal. All untreated MPS I dogs had a facial dysmorphic score of 3 (appeared severely affected) at 7 months or older ($N = 5$). In contrast, RV-treated dogs had a dysmorphic score of 0 (appeared normal) at all ages of evaluation, as shown for representative examples in **Figure 1c** and **d** ($N = 6$; $P < 0.001$ for MPS I versus RV-treated). Four of five untreated MPS I dogs had thickening and prolapse of the third eyelid (**Figure 1f**), a triangular structure that sometimes partially covers the lower medial part of the eye and contains cartilage and a tear gland. The third eyelid was normal in all RV-treated dogs (**Figure 1g** and **h**).

Corneal clouding

Corneal clouding was scored from 0 (normal) to 3 (severe). Corneal clouding in untreated MPS I dogs resulted in a blurry view of the fundus (**Figure 1j**) and an orange peel–like corneal texture due to granular opacities (**Figure 1n**). Corneal clouding was scored at 2 ± 0 at both 6 and 12 months in untreated MPS I dogs ($N = 4$) and was markedly reduced in the RV-treated dogs regardless of the serum IDUA activity (**Figure 1k**, **l**, **o** and **p**), with scores of 0.9 ± 0.5 at 6 months ($N = 5$; $P = 0.005$) and 0.6 ± 0.5 at 12 months ($N = 6$; $P = 0.001$).

Organ lysosomal enzyme and GAG levels

In a previous study, liver had the highest expression after neonatal RV-mediated gene therapy to MPS VII dogs, although spleen and blood cells had 33 and 2% as much RV RNA as liver after normalization to β-actin levels.[24] Liver biopsies obtained at 4 months from RV-treated MPS I dogs had 428 ± 425 U/mg of IDUA activity, which was 15-fold the value of 27 ± 8 U/mg in normal dogs (**Figure 2a**). The serum IDUA activity was directly proportional to the liver IDUA activity for individual dogs. Untreated MPS I dogs had very low liver IDUA activity at 1.1 ± 0.2 U/mg.

Organs from post-mortem samples were evaluated for IDUA activity for two RV-treated dogs with low serum IDUA activity (I-99 and I-101) and two RV-treated dogs with high serum IDUA activity (I-107 and I-140), and white blood cells (WBCs) were analyzed from two RV-treated dogs with high serum IDUA activity (I-171 and I-172). Liver IDUA activity remained high at 300 ± 384 U/mg (**Figure 2a**). The percentage normal IDUA activity in RV-treated dogs was 1,000% in spleen, 80% in brain, 30% in heart, 230% in kidney medulla, 210% in kidney cortex, 20% in aorta, and 56% in WBC. MPS I results in an elevation

Table 1 Summary of untreated and RV-treated MPS I dogs

Dog	Name	Age[a] (mo)	Vector dose[b] ×10[9]	Serum IDUA[c] (U/ml)	Comments	Cause of death
Untreated MPS I dogs						
I-113	Tsunami	14	None	0.6 at 4 mo	Umbilical hernia, short stature, mild chest deformity, carpi varus, weak and hyperreflexic in all four limbs	Euthanized due to neurological disease
I-115	Halle	7	None	0.4 at 6.5 mo	Umbilical hernia, carpi valgus, phalangeal joint laxity, elbow effusion	Planned euthanasia
I-119	Denzel	12	None	0.6 at 6 mo	Umbilical hernia, carpi valgus, phalangeal joint laxity, ataxia, weak and hyperreflexic in hind limbs	Euthanized due to neurological disease
I-120	Rudy	21	None	0.5 at 8 mo	Umbilical hernia, moderate elbow effusion and crepitus, mild phalangeal joint laxity	Planned euthanasia
I-137	Cairo	15	None	Not tested	Umbilical hernia, moderate chest deformity	Planned euthanasia
Average ± SD			None	0.5 ± 0.1 $N = 4$		
RV-treated MPS I dogs						
I-96	Enola	3	0.68	57 ± 36	Too young for evaluation	Unknown cause
I-99	Glennis	13	3.7	23 ± 6	Luxated shoulders, otherwise normal	Planned euthanasia
I-101	Belle	21	3.4	27 ± 12	Appears normal	Planned euthanasia
I-107	St. Louis	20	2.8	553 ± 263	Appears normal	Planned euthanasia
I-108		2 d.	2.8		Too young for evaluation	Unknown cause
I-136	Spitfire	0.25	9.9	1221 ± 332	Too young for evaluation	Unknown cause
I-140	Schmidt	15	10.1	888 ± 546	Appears normal, closed umbilical hernia	Planned euthanasia
I-171	Tom	18	2.6	240 ± 138	Appears normal	Still alive
I-172	Dick	18	2.5	608 ± 222	Appears normal	Still alive
Average ± SD			4.3 ± 3.4	454 ± 444		
Normal dogs						
Average ± SD				13.0 ± 1.9 $N = 6$		

Abbreviations: MPS I, mucopolysaccharidosis I, RV, retroviral vector; IDUA, α-L-iduronidase.
[a]At time of death or current age at time of writing (I-171 and I-172) in months except for I-108, where values are given in days. [b]Dose of RV in transducing units/kg.
[c]Lifetime mean ± SD serum IDUA activity is listed for RV-treated dogs; value at the stated age in months is listed for untreated dogs.

of other lysosomal enzymes, and correction of disease normalizes these values. Organs of untreated MPS I dogs had total β-hexosaminidase levels 2.1- to 4.6-fold the levels in normal dogs ($P < 0.01$, **Figure 2b**), although values in WBC were only 1.3-fold normal. The RV-treated dogs had β-hexosaminidase levels 1.0- to 1.9-fold normal, which were lower ($P < 0.05$) than values in untreated MPS I dogs in all sites except WBC. MPS I results in accumulation of soluble sulfated GAGs, and correction of disease normalizes these values. Untreated MPS I dogs had organ GAG levels that were 17- to 108-fold normal (**Figure 2c**; $P < 0.01$), although values in WBC were only four-fold normal. GAG levels were reduced to 1.0- to 2.6-fold normal in RV-treated dogs (not significant versus normal), which was significantly lower than values in untreated MPS I dogs ($P < 0.01$) in all sites except WBC.

Heart

Figure 3a shows representative images of echocardiograms obtained at 1 year, and **Figure 3b** shows average scores for echocardiogram-derived parameters at 6 and 12 months. Untreated MPS I dogs had mild aortic dilatation, with a diameter of 40.1 ± 9.8 mm/m^2 body surface area at 12 months. At this age, RV-treated dogs had an average aorta diameter of 24.5 ± 4 mm/m^2 ($P = 0.003$ versus untreated MPS I dogs), which was similar to values in normal dogs (not shown). At 12 months, untreated MPS I dogs had a mild subjective aortic dilatation score of 1.0 ± 0.8 on a scale of 0 (normal) to 4 (severely abnormal), which was significantly higher than the value of 0 ± 0 ($P = 0.015$) in RV-treated dogs. Aortic valve thickening and aortic insufficiency were mild, and there were no significant differences between the groups at 12 months.

There were moderate amounts of lysosomal storage (severity score 2.0 ± 0 on a scale from 0 (normal) to 3 (severe)) and mild elastic fiber fragmentation (severity score on the same scale of 1.8 ± 0.5) throughout the layers of the media of the aortas from untreated MPS I dogs (**Figure 3c**). Aortas from three of four RV-treated dogs appeared completely normal, whereas the aorta from one RV-treated dog with low serum IDUA activity (I-101) had mild (score = 1) storage and elastin fragmentation in

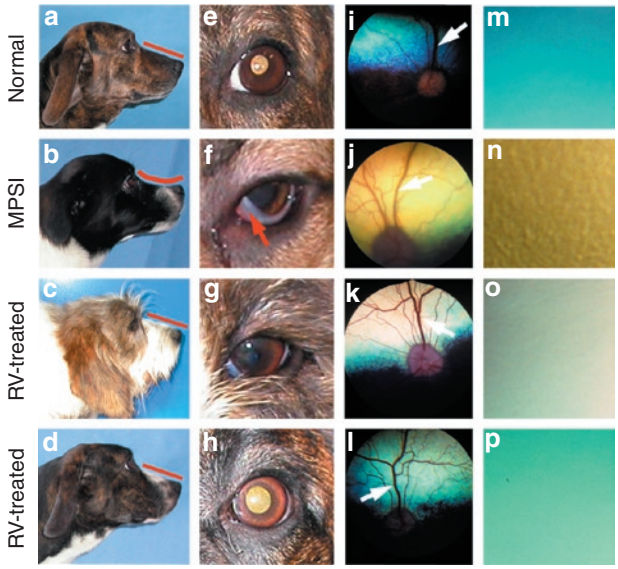

Figure 1 Facial dysmorphia and corneal clouding. Retroviral vector (RV)–treated mucopolysaccharidosis I (MPS I) dogs were injected with ~4 × 10^9 transducing units/kg of hAAT-cIDUA-WPRE at 2–3 days after birth. (**a–d**) Facial profiles. (**b**) Photographs obtained at 1 year of age show the pronounced scoop in the muzzle in an untreated MPS I dog, which is emphasized by the red line drawn above it. (**a**) A normal dog and RV-treated MPS I dogs with (**c**) low or (**d**) high serum α-L-iduronidase (IDUA) activity had normal muzzle profiles. (**e–h**) Third eyelid. (**f**) Photographs obtained at ~1 year of age demonstrate the prolapse of the third eyelid (membrana nictitans) of an untreated MPS I dog, as indicated with a red arrow. The prolapse is absent in (**e**) the normal and in RV-treated MPS I dogs with (**g**) low and (**h**) high serum IDUA activity. (**i–l**) Corneal clouding. (**j**) An untreated MPS I dog had a blurred appearance to the retinal blood vessels (white arrows) owing to the corneal clouding at ~1 year of age. (**i**) A normal dog and (**k, l**) both RV-treated dogs with low serum IDUA activity had well-defined blood vessels because the corneas had little or no lysosomal storage. (**m–p**) Corneal surface. (**n**) Retroillumination at ~1 year of age demonstrates the granular "orange peel" appearance of the cornea of an untreated MPS I dog, which indicates corneal storage. (**m**) The cornea appears smooth in a normal and (**o, p**) both RV-treated dogs with low serum IDUA activity. The color difference is due to normal variation in the color of the tapetum lucidum and is not due to treatment or disease.

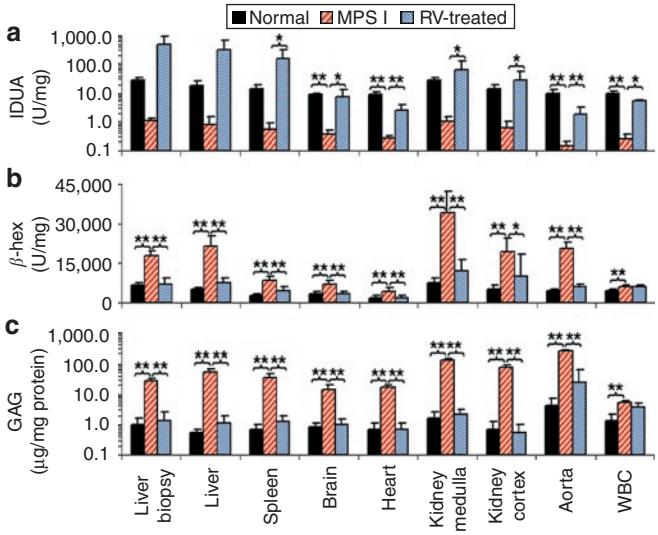

Figure 2 Organ lysosomal enzyme and glycosaminoglycans (GAG) levels. Liver biopsies were obtained from retroviral vector (RV)–treated dogs at 4 months of age (N = 6). Samples from other organs were obtained at post-mortem at 13–21 months after birth from two RV-treated dogs with low expression (I-99 and I-101) and two RV-treated dogs with high expression (I-107 and I-140). Organs from homozygous normal dogs (N = 8) and untreated mucopolysaccharidosis I (MPS I) dogs (N = 5) were collected at similar ages. White blood cells (WBCs) were obtained from two RV-treated dogs with high serum α-L-iduronidase (IDUA) activity at 17 months (I-171 and I-172) and from normal (N = 6) and MPS I (N = 4) dogs at 4 to 12 months of age. Means ± SD are shown, * indicates a P value of 0.01–0.05 and ** indicates a P value <0.01 for comparison of values in the groups that are connected with a bracket using ANOVA with Tukey post hoc analysis. (**a**) IDUA activity. IDUA activity was determined in organ homogenates and normalized to the amount of protein in the extract. (**b**) β-Hexosaminidase (β–hex). Total β–hex activity was determined as in **a**. (**c**) GAG levels. Sulfated GAG levels were determined.

the middle region of the aortic media. This resulted in an overall score of 0.25 ± 0.5 for both of these parameters in RV-treated dogs, which was significantly lower (P < 0.01) than in untreated MPS I dogs.

Mitral valve thickening analysis is shown in the bottom panels of **Figure 3a** and is quantified in **Figure 3b**. Untreated MPS I dogs had moderate mitral valve leaflet thickening, with severity scores of 1.6 ± 0.5 and 1.5 ± 0.6 at 6 and 12 months, respectively. Mitral valve thickening scores were markedly reduced in the RV-treated dogs at 0.5 ± 0.6 (P = 0.009) and 0.3 ± 0.5 (P = 0.01) at 6 and 12 months, respectively. Mild mitral valve insufficiency was present in both groups and also in some normal controls, and there were no significant differences between untreated and RV-treated MPS I dogs.

Neurological disease

Cognitive impairment was not obvious in untreated MPS I dogs: they displayed normal canine behavior, chased balls, and responded to their names. Human patients can have spinal cord compression from bone and vertebral disc disease or thickening of the meninges. Indeed, one untreated MPS I dog (I-113) with narrowing of the C5/C6 intervertebral disc space on radiographs (data not shown) was quadraparetic and had proprioceptive deficits and hyperreflexia in all four limbs at 14 months. A second dog (I-119) showed pelvic limb proprioceptive deficits and hyperreflexia, suggesting spinal cord disease between T3 and L3, although disc space narrowing was not noted on radiographs. Both dogs were euthanized for humane reasons at the onset of these signs. Signs of spinal cord disease were absent in the RV-treated MPS I dogs. In addition, the untreated MPS I dog I-119 developed nystagmus and ataxia of the head and limbs at 12 months, suggesting cerebellar and/or vestibular system involvement. Ataxia was not seen in any RV-treated dogs.

Pathology of the brain

Brains were evaluated histologically for evidence of lysosomal storage. All untreated MPS I dogs had numerous large dark granules in most cortical neurons when 1-μm sections were stained with toludine blue (**Figures 4b** and **f**). The dark color was similar to that seen in the synovial cells of MPS I dogs[25] but differed from the clear vacuoles previously observed in brains of MPS I mice,[15] MPS VII mice,[26] or MPS VII dogs.[24] Electron microscopy confirmed that the dark granules in

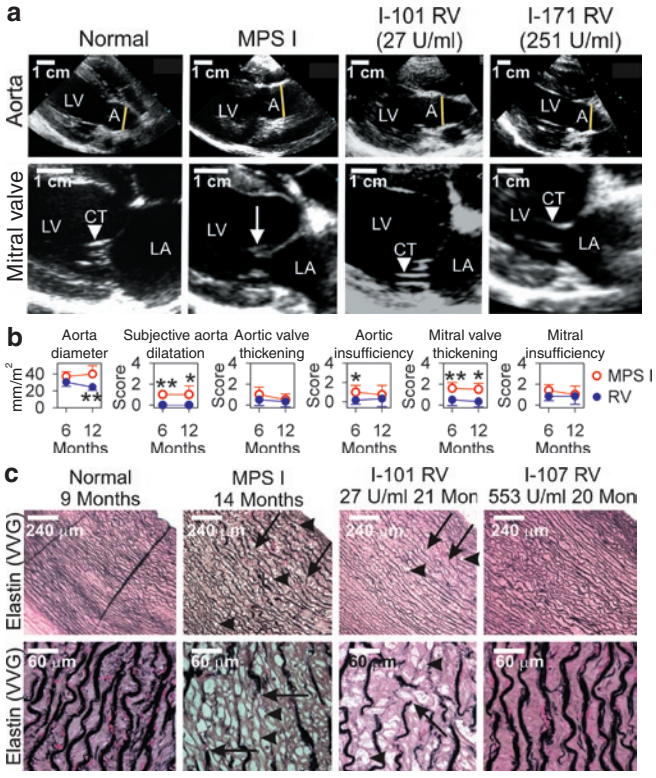

Figure 3 Amelioration of heart and aorta disease. (**a**) Echocardiograms. Right parasternal long-axis echocardiograms were performed on normal, untreated mucopolysaccharidosis I (MPS I), or retroviral vector (RV)–treated MPS I dogs with low (I-101) or high (I-171) serum α-L-iduronidase activity. The top panels show the left ventricular (LV) outflow tract. The line across the aorta (A) indicates the approximate position of the conotubular junction where the long-axis diameter was measured. Note the normal narrowing at the right side of the aorta in the normal and both RV-treated MPS I dogs, which was absent in the untreated MPS I dog. The bottom panels show the left atrium (LA), mitral valve apparatus, and LV. The insertion of normal chordae tendinae (CT) into the mitral valve leaflets is indicated with white arrowheads. In the untreated MPS I dog, the chordae tendinae and tips of the mitral valve leaflets are thickened and nodular, as indicated by the long white arrow. Scale bar = 1 cm. (**b**) Quantification of the echocardiograms. Echocardiograms were performed at 6 and 12 months after birth on four untreated MPS I and six RV-treated MPS I dogs. In the left panel, the long-axis diameter of the aorta was normalized to the body surface area in square meters (m^2), and the mean ± SD determined. Values in the two groups were compared with the Student's *t*-test; * indicates a *P* value of 0.01–0.05, and ** indicates a *P* value <0.01. In the other panels, the parameter was scored from 0 (normal) to severely abnormal (4), and the average scores were determined and compared statistically. (**c**) Pathology of the aorta. Aortas were collected at the age indicated above the panels and then stained for elastin. Lysosomal storage is identified with black arrowheads, and fragmentation of the elastin fibers is identified with long black arrows. Scale bar = 240 μm for the top panels and 60 μm for the bottom panels. AI, aortic insufficiency; AV, aortic value; MI, mitral insufficiency; MV, mitral value; thick, thickening.

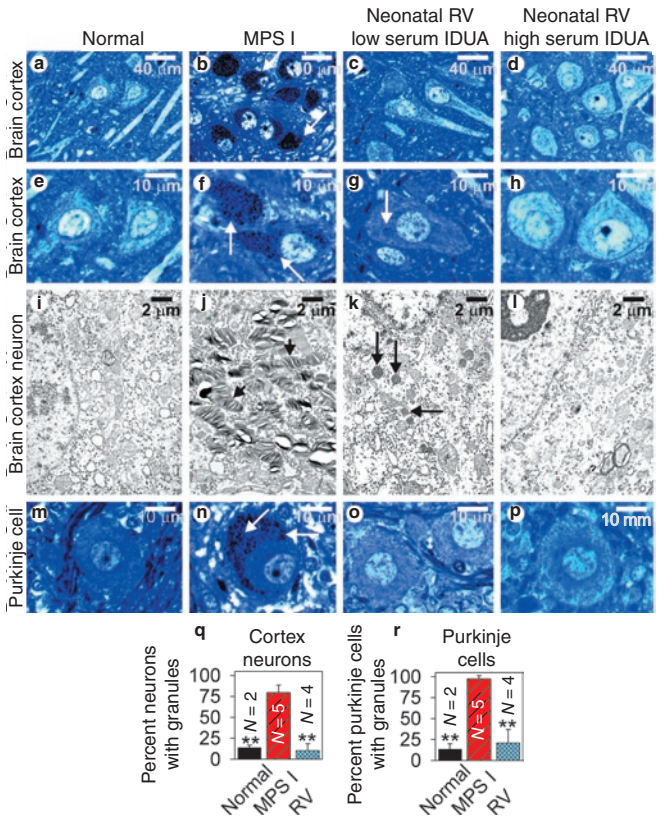

Figure 4 Evaluation of brains for lysosomal storage. Samples were collected at 1–1.5 years. (**a–h**) Light microscopy of the frontal cortex. The groups are indicated above the panels. Sections 1 μm thick were stained with toluidine blue. The white arrows indicate dark granules in the cytoplasm of neurons, which are consistent with lysosomal storage. Scale bar = 40 μm (**a–d**) and 10 μm (**e–h**). (**i–l**) Electron microscopy of the frontal cortex. The short black arrows identify zebra bodies in cortical neurons, which are consistent with lysosomal storage. The long black arrows identify small lysosomes that were apparent in a small percentage of the neurons from an retroviral vector (RV)–treated dog with low serum α-L-iduronidase (IDUA) activity. Scale bar = 2 μm. (**m–p**) Light microscopy of Purkinje cells. Sections 1 μm thick were stained with toluidine blue. The white arrows indicate dark granules in the cytoplasm of Purkinje cells, which are consistent with lysosomal storage. Scale bar = 10 μm. (**q–r**) Percentage of cells with storage in neurons of the frontal cerebral cortex or Purkinje cells. The percentage of cells with lysosomal storage was determined for cortical neurons and Purkinje cells for the indicated number of animals, and the mean ± SD was determined. Statistical comparisons were performed as described in **Figure 2**. MPS I, mucopolysaccharidosis I.

cortical neurons from untreated MPS I dogs were zebra bodies (**Figure 4j**) or large lysosomes with circular whorls (not shown), which have previously been reported to represent lysosomal storage in patients with MPS I.[1] All RV-treated MPS I dogs had a remarkable reduction in the amount of lysosomal storage in the cortical neurons as assessed with light microscopy regardless of the serum IDUA activity. In addition, zebra bodies and large lysosomes with circular whorls were absent when electron microscopy was performed on brain cortex from one RV-treated dog with low and one with high serum IDUA activity. However, the dog with low serum IDUA activity did have occasional cells with increased numbers of small lysosomes (**Figure 4k**), which may represent modest amounts of lysosomal storage. Untreated MPS I dogs had five or more granules that were consistent with lysosomal storage in 79 ± 9% of cortical neurons (**Figure 4q**), which was significantly higher ($P < 0.01$) than the percentage of cells with granules in normal (14 ± 3%) or RV-treated (11 ± 8%) dogs. Similarly, untreated MPS I dogs had vacuoles consistent with lysosomal storage in 98 ± 4% of Purkinje cells (**Figure 4n** and **r**). This was significantly higher ($P < 0.01$) than the values of 14 ± 6% in normal dogs and of 17 ± 12% in

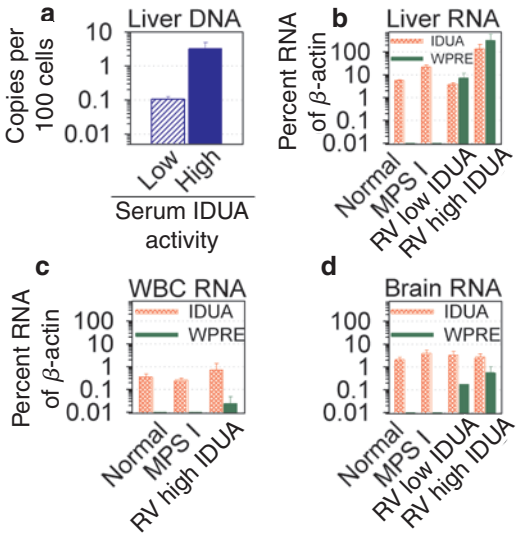

Figure 5 Retroviral vector (RV) DNA and RNA. (**a**) RV DNA. RV DNA copies were determined with real-time polymerase chain reaction (RT-PCR) for the woodchuck hepatitis post-transcriptional regulatory element (WPRE) of the RV with normalization to β-actin. DNA was evaluated in liver of two RV-treated mucopolysaccharidosis I (MPS I) dogs with low serum α-L-iduronidase (IDUA) activity and two RV-treated dogs with high serum IDUA activity at 4–21 months after birth. Samples from normal and untreated MPS I dogs had no signal (not shown). (**b**) Liver RV RNA. Liver RNA was evaluated by real-time RT-PCR for canine IDUA RNA levels with primers that amplified both the endogenous and the RV-derived RNA, for RV sequences with primers specific for the WPRE, and for β-actin sequences for normalization. The amount of IDUA or WPRE RNA relative to the β-actin signal was determined for two samples from each group. (**c**) White blood cells (WBCs) RV RNA. WBC was evaluated as in **b** for two RV-treated MPS I dogs with high serum IDUA activity at 17 months and also for MPS I ($N = 4$) and normal ($N = 4$) dogs at 4–12 months. (**d**) Brain RV RNA. RNA from the frontal cortex of the brain was analyzed for IDUA, WPRE, and β-actin sequences for two samples from each group for most assays and for three samples from MPS I dogs.

RV-treated dogs. In addition, untreated MPS I dogs had numerous clear vacuoles that were consistent with lysosomal storage in the meninges and perivascular cells, as reported previously,[27] which were absent in all RV-treated dogs (data not shown).

Vector DNA and RNA in liver, blood cells, and brain

The RV DNA copy number was evaluated in liver using real-time polymerase chain reaction (real-time PCR) for the woodchuck hepatitis post-transcriptional regulatory element (WPRE) of the RV with normalization to β-actin levels (**Figure 5a**). Livers from RV-treated dogs with low and high serum IDUA activity had 0.11 ± 0.02 and 3.2 ± 1.5 copies of RV per 100 cells, respectively. This suggests that the 21-fold differences in serum IDUA activity between the two groups may be due to the 29-fold variation in liver transduction efficiency. RV DNA was present at low levels in WBC of RV-treated dogs with high serum IDUA activity but was not analyzed in RV-treated dogs with low serum IDUA activity. Similarly, RV DNA was present at low levels in brain of RV-treated dogs with low and high serum IDUA activity. However, it was not possible to quantify the amount of DNA in either WBC or brain because the C_T for the WPRE was high (35–38), which is a level where the standard curve is not linear in our hands. The DNA concentration was estimated to be less than 0.1 copy per 100 cells in both organs. WBC and brain from non-transduced MPS I or normal dogs had WPRE C_T values of 40, demonstrating the specificity of the real-time PCR for RV sequences (data not shown).

RNA was evaluated with IDUA primers that amplified the endogenous as well as the RV-derived canine IDUA complementary DNA (cDNA), with WPRE primers specific for the RV-derived RNA, and with β-actin primers for normalization. The liver IDUA signal in normal and MPS I dogs was, respectively, $5.6 \pm 0.4\%$ and $22 \pm 5\%$ of the β-actin signal (**Figure 5b**), demonstrating that untreated MPS I dogs had fourfold more IDUA RNA in liver than normal dogs. As expected, the liver WPRE signal was undetectable in both normal and untreated MPS I dogs. RV-treated dogs with low serum IDUA activity had 67% of normal liver IDUA RNA levels, which was less than the background in untreated MPS I dogs. However, the liver WPRE RNA was $7.4 \pm 4.0\%$ of β-actin, indicating that IDUA expression was 132% of normal—assuming that the IDUA and the WPRE primer sets amplified equally well. This was consistent with the liver IDUA activity of 110% of normal in these RV-treated dogs with low serum IDUA activity. RV-treated dogs with high serum IDUA activity had IDUA RNA levels that were $135 \pm 78\%$ of β-actin (24-fold normal) and WPRE RNA levels that were $323 \pm 277\%$ of β-actin (58-fold normal, assuming the IDUA and WPRE primers amplified equally well). The RNA levels in RV-treated dogs with high serum IDUA activity were consistent with liver IDUA activity that was 32-fold normal.

RNA was also evaluated from WBC (**Figure 5c**) and cerebral cortex (**Figure 5d**) to determine whether RV expression in these sites might have contributed to the correction of lysosomal storage in RV-treated dogs. MPS I dog WBC and brain had 74 and 142% as much IDUA RNA as in normal dogs, respectively, and did not have a signal for the WPRE. The WPRE signal in WBC of RV-treated dogs with high serum IDUA activity was $0.02 \pm 0.02\%$ of β-actin, which was 6% of the level in normal WBC if one assumes that the WPRE and IDUA primers amplify equally well. WBC RNA was not evaluated in RV-treated dogs with low serum IDUA activity. The WPRE signal in brain of RV-treated dogs with low or high serum IDUA activity was 0.2 ± 0.002 or $0.5 \pm 0.5\%$ of β-actin, respectively. This indicates that RV-treated dogs with low or high serum IDUA activity had 10 or 24% as much IDUA RNA in brain, respectively, as normal dogs if the IDUA and WPRE primers amplified equally well.

DISCUSSION

This study demonstrates that many clinical manifestations were prevented in MPS I dogs that achieved stable IDUA expression after neonatal IV injection of an RV. The RV-treated dogs with low and high serum IDUA activity had 25 ± 3 U/ml (2-fold normal) and 535 ± 285 U/ml (41-fold normal) of IDUA activity in serum, respectively. The major source of enzyme in blood was probably the liver, which we previously demonstrated was the organ with the highest expression after a similar gene therapy approach in MPS VII dogs.[24] The lack of overt adverse effects from very high serum IDUA activity is likely due to the fact that IDUA functions best at a low pH and acts only upon terminal iduronic acids in GAG chains.

Lack of an immune response after neonatal gene therapy

One reason for using gene therapy in MPS I dogs was to assess the immune response in a large-animal model with a null mutation, as such mutations are common in Hurler syndrome patients.[28] Serum from these RV-treated dogs did not have anti-canine IDUA immunoglobulin G antibodies in an enzyme-linked immunosorbent assay in which wells that were coated with canine IDUA were used to capture antibodies (data not shown). This was somewhat surprising given that MPS I dogs mounted an antibody response to canine IDUA protein after ERT[29] and to canine IDUA after HSC- or myoblast-directed gene therapy.[18–20] The immaturity of the immune response at birth may have contributed to this failure to produce anti-canine IDUA antibodies. Alternatively, the lack of antibodies could be due to the high serum levels of canine IDUA, as animals with 25 U/ml have ~100 ng/ml or ~2 nmol/l of serum canine IDUA, assuming a specific activity of 250,000 U/mg.[30] This concentration was effective at preventing an antibody response to human Factor IX after neonatal gene therapy.[31]

The stable expression in RV-treated dogs indicates indirectly that a cytotoxic T-lymphocyte response did not destroy transduced cells. This result differs from that in MPS I cats that received the same canine IDUA-expressing RV as newborns and mounted a potent cytotoxic T-lymphocyte response.[23] Although we previously hypothesized that the newborn immune system may be less mature in dogs than in cats, it is also possible that MPS I dogs express canine IDUA epitopes and develop tolerance. Indeed, IDUA messenger RNA was near-normal in untreated MPS I dogs in this study, and substantial amounts of an IDUA cDNA were amplified from blood cells of untreated MPS I dogs with exon 3 and exon 6 primers in a previous study.[20] The finding of near-normal levels of IDUA RNA in untreated MPS I dogs in this study differs from the result in fibroblasts from MPS I dogs, where levels of a 2.8 kilobase RNA (which was hypothesized to include the 450 bp intron 1 and sequences from the wild-type 2.2 kilobase messenger RNA) were both low.[2,3] The 5′ splice site mutation results in a stop codon within intron 1 at nucleotide 519 (GI:163964) and a truncated 52 amino acid protein, yet translational initiation at the second AUG at nucleotide 942 within intron 1 would be in frame with exon 2 (starts at nucleotide 968) and would produce a protein with MPAPSLCPS in addition to the sequence encoded by exons 2–14. This putative protein lacks a signal sequence and should not be secreted or transported to the lysosome, but it could result in epitope presentation and tolerance. Although it was previously proposed that *ex vivo* gene therapy of HSC[19,20] or myoblasts[18] may have resulted in a cytotoxic T-lymphocyte response to canine IDUA, these data were indirect. Further studies with the human IDUA gene in the MPS I dogs may help to determine whether the prolonged expression observed in this study was due to the immaturity of the newborn canine immune system or to tolerance to canine IDUA epitopes.

Heart disease

Cardiac disease can result from storage in heart values, aorta, coronary arteries, and/or myocardium. In this study, untreated MPS I dogs had moderate thickening of the mitral valve, which was markedly reduced in all RV-treated dogs regardless of the serum IDUA activity. Similarly, heart valve thickening stabilized or was reduced after HSCT or ERT. Untreated MPS I dogs had aortic diameters that were mildly dilated at 164% of normal at 1–1.5 years of age, which was associated with moderate amounts of lysosomal storage and mild elastic fiber fragmentation throughout the thickness of the media. Pathology in dogs was similar to that in untreated humans but less severe than in MPS I mice.[14,32–34] This difference in severity may result from less lysosomal storage per cell in the aorta of large animals than in mice. Both RV-treated dogs with high serum IDUA activity (553 and 888 U/ml) had complete correction of aortic disease, which was consistent with the ability of neonatal gene therapy to correct aortic disease in mice if serum IDUA activity is >500 U/ml.[14] Although one RV-treated dog with low serum IDUA activity (23 U/ml) that was killed at 13 months had complete pathological correction in the aorta, the other RV-treated dog with low serum IDUA activity (27 U/ml; I-101) that was euthanized at 21 months had mild lysosomal storage and elastic fiber fragmentation in the middle regions of the media. The failure to prevent aortic disease in I-101 is consistent with the results in MPS I mice, where administration of a low dose of RV to neonatal mice resulted in only ~30 U/ml of serum IDUA activity.[14] Difficulties in preventing aortic disease were also encountered with other treatments: aortic regurgitation developed *de novo* in eight of nine patients at 12 years after performing HSCT at a mean age of 2 years;[7] and 60% of patients analyzed at 4–6 years after initiation of ERT had *de novo* development or worsening of aortic insufficiency.[11,13] Evaluating aortas from additional dogs with low and high serum IDUA activity at older ages will be important.

Neurological disease

A major problem in children with Hurler syndrome is mental retardation, which is likely due in part to neuronal lysosomal storage. In this study, all RV-treated dogs had marked reductions in lysosomal storage in cortical neurons even when serum IDUA activity was only ~25 U/ml. This differed from the result in MPS I mice, where animals with ~30 U/ml after low-dose neonatal gene therapy had substantial lysosomal storage in neurons.[15] One difference may be that the brain of RV-treated MPS I dogs with low serum IDUA activity had 10% as much IDUA messenger RNA as did normal dog brain; this was much higher than the level of RV RNA found in brain in MPS I mice with low serum IDUA activity. Expression in brain of RV-treated dogs presumably derived from the LTR, which can drive expression of a translatable protein in non-hepatic cells.

The presence of relatively high levels of RV RNA sequences in the brain of RV-treated MPS I dogs at 1 year could be due either to transduction of brain at the time of IV injection of vector or to transduction of hematopoietic cells that migrated into brain. Analysis of DNA was not helpful at differentiating between these possibilities, because the signal was too low in both WBC and brain of RV-transduced dogs for meaningful quantitation. That RV RNA in brain was as high or higher as in WBC in this study could suggest that brain cells were transduced, but it is also possible that the LTR was up-regulated in blood-derived cells in brain. We similarly reported that DNA was present in the brain of MPS VII dogs at 6 months after neonatal gene therapy,

although RNA could not be evaluated because it was of poor quality.[24] In contrast, we previously reported that normal dogs receiving an amphotropic RV that expressed β-galactosidase from the LTR had undetectable protein and DNA in brain at 1 week after neonatal transduction.[35] This apparent discrepancy may be due to differences in the permeability of the blood–brain barrier in MPS I or MPS VII as compared with normal dogs, to a low sensitivity for protein expression and DNA copies in the previous study with β-galactosidase, or to an ability of transduced WBC or neuronal cells to increase their relative representation in the brain over time. No expressing cells were identified in the brain parenchyma using a histochemical stain at 6 months after neonatal transduction with a β-glucuronidase-containing RV in MPS VII dogs, suggesting that the expression per cell was relatively low.[24] Future studies will attempt to identify the cell types that express the RV in the brain using a green fluorescent protein–expressing vector. If expression in the brain is, indeed, the critical factor for reducing lysosomal storage in the brain, then utilization of a self-inactivating vector to improve the safety[36] might reduce the efficacy of correction in brain. We will therefore also test whether a self-inactivating vector that does not express in brain is effective.

It remains possible that brain was corrected because IDUA protein in blood crossed the blood–brain barrier. Indeed, a recent report showed that IV injection of high doses of β-glucuronidase protein into MPS VII mice reduced lysosomal storage in neurons.[37] Furthermore, transduction of liver cells in adult MPS I mice reduced lysosomal storage in brain without resulting in detectable RNA,[38] and neonatal administration of an adeno-associated vector reduced storage in the cerebellum and improved neurological function with no detectable vector copies in brain.[17] The ability to treat disease in the brain with IV injection of RV is important because some have argued that localized injections, as recently reported by Ciron *et al*.[39] in MPS I dogs, are necessary to achieve improvement in the brain.

Implications for gene therapy for MPS I in children

The findings of this study appear promising for future therapy of children with MPS I using neonatal IV injection of an IDUA-expressing RV. It is likely that the benefits would be maintained, as MPS VII dogs that received a similar neonatal gene therapy procedure[24,40–42] remain alive and well with high serum β-glucuronidase activity at 6 years. Additional studies will determine the mechanism of improvement in the brain by testing whether self-inactivating vectors that express only in the liver are equally effective. The possibility of neoplasia from insertional mutagenesis remains a major concern for this approach. Although this risk appears to be low, we are currently identifying integration sites and testing for clonal expansion in RV-treated mice and dogs that received this neonatal IV injection procedure. A self-inactivating vector that lacks the enhancer region of the LTR[36] should reduce this risk and will be utilized if it maintains efficacy for treating brain disease. Although immune responses did not occur in these dogs, such a response remains a concern for patients with null mutations, as MPS I cats developed a cytotoxic T lymphocyte response after neonatal administration of an RV expressing the canine IDUA cDNA.[23] Finally, this approach requires that patients can be identified at birth. Recent progress in techniques for newborn diagnosis of LSD including MPS I appears to be promising,[43,44] making it likely that patients will be identified early enough in the near future to initiate a gene therapy treatment similar to the one in this study. If the concerns raised here can be addressed, then gene therapy may become a possibility for treatment of children with MPS I.

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise described.

Retroviral vector. The RV designated hAAT-cIDUA-WPRE containing intact LTRs, the human α_1-antitrypsin (hAAT) promoter, the canine IDUA cDNA, and the WPRE was prepared as previously described and was devoid of replication-competent retrovirus.[14]

MPS I dogs. MPS I dogs generously provided by Dr. Emil Kakkis were maintained at the School of Veterinary Medicine, University of Pennsylvania, under NIH and USDA guidelines for the care and use of animals in research. Dogs were outbred into a colony of mixed-breed dogs to increase their reproductive performance and overall vigor. MPS I dogs were identified at birth by serum IDUA assay and confirmed by DNA testing. RV-treated MPS I dogs were injected with 2–3 doses of a 5–8 ml bolus of hAAT-cIDUA-WPRE over 1–2 minutes at 4-hour intervals via an external jugular vein catheter at 2–3 days after birth. Liver biopsies, radiographs, eye exams, echocardiograms, euthanasia, and organ collection were performed as previously described.[35] Dogs were not perfused before the collection of tissues.

Organ IDUA, β-hexosaminidase, and GAG levels. Post-mortem tissues were analyzed for IDUA activity, β-hexosaminidase activity, and GAG levels as previously described.[14,23,24]

Pathology. Brain samples were fixed with 4% paraformaldehyde and 2% gluteraldehyde in phosphate-buffered saline, embedded with Epon (Electron Microscopy Sciences, Fort Washington, PA), and 1-μm sections were stained with toluidine blue. At least 50 cells were scored for five or more dark blue granules that were consistent with lysosomal storage. Aorta samples were fixed in the same fashion but were embedded in paraffin, and 4-μm sections were stained with Vierhof van Giesen stain for elastin.

Vector DNA and RNA in organs. DNA was isolated from liver and RNA was isolated from blood cells and organs as reported previously.[23,24] DNA copies were determined using Taqman PCR for the WPRE with normalization to β-actin as described previously.[35] DNA samples with high β-actin C_T values were excluded from analysis. RNA was reverse transcribed with a Superscript III kit (Invitrogen, Carlsbad, CA) and a mixture either of reverse β-actin and reverse canine IDUA primers or of reverse β-actin and reverse WPRE primers. IDUA primers were from nucleotide 1788 to 1811 (5′-CAAGTGCCTGTGGACCTATGAGAT-3′) of the canine IDUA cDNA, which contains 3 nucleotides of exon 12 and 21 nucleotides of exon 13, and from the complement of nucleotides 1870–1847 (5′-TAAAGGTG GATGGCTTCCTGCTGA-3′) from exon 13. The Taqman probe was from nucleotide 1846 to 1817 of exon 13 (5′-TCTCCGCGGATGGAGAAGT GTACACGCCCA-3′). Real-time PCR was then performed with canine IDUA–specific reagents with normalization to β-actin or with WPRE specific reagents with normalization to β-actin using a Taqman PCR core reagent kit (Applied Biosystems, Foster City, CA).[35] The amount of IDUA or WPRE RNA relative to the β-actin signal was determined by comparison of the cycle number to reach the threshold (C_T).

Statistical evaluations. The mean ± the SD was calculated for all values, and comparisons were between two groups using the Student's *t*-test or between multiple groups using ANOVA with Tukey post hoc analysis.

ACKNOWLEDGMENTS

We thank Emil Kakkis (Biomarin) for sending us mucopolysaccharidosis I dogs and canine α-L-iduronidase protein, Elizabeth Neufeld (University of California, Los Angeles) for several helpful conversations, Ralph Commons (Michigan State University) for help with pathology, and Clay Semenkovich and Trey Coleman (Washington University) for assistance with real-time PCR. This work was supported by the Ryan Foundation, the National MPS Society, and the National Institutes of Health (DK66448 awarded to K.P.P., EY-13132 awarded to G.D.A., and RR02512 awarded to M.H.). Real-time PCR was supported by the Phenotyping Core of the Diabetes Research and Training Center (DK20579) awarded to Clay Semenkovich.

REFERENCES

1. Neufeld, EF and Muenzer, J (2001). The mucopolysaccharidoses. In: Scriver, CR, Beaudet, AL, Sly, WS and Valle D (eds). *Metabolic and Molecular Basis of Inherited Disease*. McGraw-Hill, New York. pp. 3421–3452.
2. Stoltzfus, LJ, Sosa-Pineda, B, Moskowitz, SM, Menon, KP, Dlott, B, Hooper, L *et al.* (1992). Cloning and characterization of cDNA encoding canine alpha-L-iduronidase. mRNA deficiency in mucopolysaccharidosis I dog. *J Biol Chem* **267**: 6570–6575.
3. Menon, KP, Tieu, PT and Neufeld, EF (1992). Architecture of the canine IDUA gene and mutation underlying canine mucopolysaccharidosis I. *Genomics* **14**: 763–768.
4. Field, RE, Buchanan, JA, Copplemans, MG and Aichroth, PM (1994). Bone-marrow transplantation in Hurler's syndrome. Effect on skeletal development. *J Bone Joint Surg Br* **76**: 975–981.
5. Guffon, N, Souillet, G, Maire, I, Straczek, J and Guibaud, P (1998). Follow-up of nine patients with Hurler syndrome after bone marrow transplantation. *J Pediatr* **133**: 119–125.
6. Peters, C, Shapiro, EG, Anderson, J, Henslee-Downey, PJ, Klemperer, MR, Cowan, MJ *et al.* (1998). Hurler syndrome: II. outcome of HLA-genotypically identical sibling and HLA-haploidentical related donor bone marrow transplantation in fifty-four children. The storage disease collaborative study group. *Blood* **91**: 2601–2608.
7. Braunlin, EA, Stauffer, NR, Peters, CH, Bass, JL, Berry, JM, Hopwood, JJ *et al.* (2003). Usefulness of bone marrow transplantation in the Hurler syndrome. *Am J Cardiol* **92**: 882–886.
8. Staba, SL, Escolar, ML, Poe, M, Kim, Y, Martin, PL, Szabolcs, P *et al.* (2004). Cord-blood transplants from unrelated donors in patients with Hurler's syndrome. *N Engl J Med* **350**: 1960–1969.
9. Ashworth, JL, Biswas, S, Wraith, E and Lloyd, IC (2006). Mucopolysaccharidoses and the eye. *Surv Ophthalmol* **51**: 1–17.
10. Kakkis, ED, Muenzer, J, Tiller, GE, Waber, L, Belmont, J, Passage, M *et al.* (2001). Enzyme-replacement therapy in mucopolysaccharidosis I. *N Engl J Med* **344**: 182–188.
11. Sifuentes, M, Doroshow, R, Hoft, R, Mason, G, Walot, I, Diament, M *et al.* (2007). A follow-up study of MPS I patients treated with laronidase enzyme replacement therapy for 6 years. *Mol Genet Metab* **90**: 171–180.
12. Wraith, JE (2005). The first 5 years of clinical experience with laronidase enzyme replacement therapy for mucopolysaccharidosis I. *Expert Opin Pharmacother* **6**: 489–506.
13. Braunlin, EA, Berry, JM and Whitley, CB (2006). Cardiac findings after enzyme replacement therapy for mucopolysaccharidosis type I. *Am J Cardiol* **98**: 416–418.
14. Liu, Y, Xu, L, Hennig, AK, Kovacs, A, Fu, A, Chung, S *et al.* (2005). Liver-directed neonatal gene therapy prevents cardiac, bone, ear, and eye disease in mucopolysaccharidosis I mice. *Mol Ther* **11**: 35–47.
15. Chung, S, Ma, X, Liu, Y, Lee, D, Tittiger, M and Ponder, KP (2007). Effect of neonatal administration of a retroviral vector expressing alpha-L-iduronidase upon lysosomal storage in brain and other organs in mucopolysaccharidosis I mice. *Mol Genet Metab* **90**: 181–192.
16. Kobayashi, H, Carbonaro, D, Pepper, K, Petersen, D, Ge, S, Jackson, H *et al.* (2005). Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. *Mol Ther* **11**: 776–789.
17. Hartung, SD, Frandsen, JL, Pan, D, Koniar, BL, Graupman, P, Gunther, R *et al.* (2004). Correction of metabolic, craniofacial, and neurologic abnormalities in MPS I mice treated at birth with adeno-associated virus vector transduction the human alpha-L-iduronidase gene. *Mol Ther* **9**: 866–875.
18. Shull, RM, Lu, X, McEntee, MF, Bright, RM, Pepper, KA and Kohn, DB (1996). Myoblast gene therapy in canine mucopolysaccharidosis. I: abrogation by an immune response to alpha-L-iduronidase. *Hum Gene Ther* **7**: 1595–1603.
19. Shull, R, Lu, X, Dube, I, Lutzko, C, Kruth, S, Abrams-Oqq, A *et al.* (1996). Humoral immune response limits gene therapy in canine MPS I. *Blood* **88**: 377–379.
20. Lutzko, C, Kruth, S, Abrams-Ogg, AC, Lau, K, Li, L, Clark, BR *et al.* (1999). Genetically corrected autologous stem cells engraft, but host immune responses limit their utility in canine alpha-L-iduronidase deficiency. *Blood* **93**: 1895–1905.
21. Lutzko, C, Omori, F, Abrams-Oqq, AC, Shull R, Li L, Lau K *et al.* (1999). Gene therapy for canine alpha-L-iduronidase deficiency: in utero adoptive transfer of genetically corrected hematopoietic progenitors results in engraftment but not amelioration of disease. *Hum Gene Ther* **10**: 1521–1532.
22. Meertens, L, Zhao, Y, Rosic-Kablar, S, Li, L, Chan, K, Dobson, H *et al.* (2002). *In utero* injection of alpha-L-iduronidase-carrying retrovirus in canine mucopolysaccharidosis type I: infection of multiple tissues and neonatal gene expression. *Hum Gene Ther* **13**: 1809–1820.
23. Ponder, KP, Wang, B, Wang, P, Ma, X, Herati, R, Wang, B *et al.* (2006). Mucopolysaccharidosis I cats mount a cytotoxic T lymphocyte response after neonatal gene therapy that can be blocked with CTLA4-Ig. *Mol Ther* **14**: 5–13.
24. Wang, B, O'Malley, TM, Xu, L, Vite, C, Wang, P, O'Donnell, PA *et al.* (2006). Expression in blood cells may contribute to biochemical and pathological improvements after neonatal intravenous gene therapy for mucopolysaccharidosis VII in dogs. *Mol Genet Metab* **87**: 8–21.
25. Shull, RM, Munger, RJ, Spellacy, E, Hall, CW, Constantopoulos, G and Neufeld, EF (1982). Canine alpha-L-iduronidase deficiency. A model of mucopolysaccharidosis I. *Am J Pathol* **109**: 244–248.
26. Xu, L, Mango, RL, Sands, MS, Haskins, ME, Ellinwood, NM and Ponder, KP (2002). Evaluation of pathological manifestations of disease in mucopolysaccharidosis VII mice after neonatal hepatic gene therapy. *Mol Ther* ***6***: 745–758.
27. Kakkis, E, McEntee, M, Vogler, C, Le, S, Levy, B, Belichenko, P *et al.* (2004). Intrathecal enzyme replacement therapy reduces lysosomal storage in the brain and meninges of the canine model of MPS I. *Mol Genet Metab* **83**: 163–174.
28. Matte, U, Yogalingam, G, Brooks, D, Leistner, S, Schwartz, I, Lima, L *et al.* (2003). Identification and characterization of 13 new mutations in mucopolysaccharidosis type I patients. *Mol Genet Metab* **78**: 37–43.
29. Kakkis, ED, McEntee, MF, Schmidtchen, A, Neufeld, EF, Ward, DA, Gompf, RE *et al.* (1996). Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I. *Biochem Mol Med* **58**: 156–167.
30. Shull, RM, Kakkis, ED, McEntee, MF, Kania, SA, Jonas, AJ and Neufeld, EF (1994). Enzyme replacement in a canine model of Hurler syndrome. *Proc Natl Acad Sci USA* **91**: 12937–12941.
31. Xu, L, Mei, M, Haskins, ME, Nichols, TC, O'donnell, P, Cullen, K *et al.* (2006). Immune response after neonatal transfer of a human factor IX-expressing retroviral vector in dogs, cats, and mice. *Thromb Res* Nov: (epub ahead of print).
32. Renteria, VG, Ferrans, VJ and Roberts, WC (1976). The heart in the Hurler syndrome: gross, histologic and ultrastructural observations in five necropsy cases. *Am J Cardiol* **38**: 487–501.
33. Jordan, MC, Zheng, Y, Ryazantsev, S, Rozengurt, N, Roos, KP and Neufeld, EF (2005). Cardiac manifestations in the mouse model of mucopolysaccharidosis I. *Mol Genet Metab* **86**: 233–243.
34. Braunlin, E, Mackey-Bojack, S, Panoskaltsis-Mortari, A, Berry, JM, McElmurry, RT, Riddle, M *et al.* (2006). Cardiac functional and histopathologic findings in humans and mice with mucopolysaccharidosis type I: implications for assessment of therapeutic interventions in Hurler syndrome. *Pediatr Res* **59**: 27–32.
35. Xu, L, Haskins, ME, Melniczek, JR, Gao, C, Weil, MA, O'Malley, TM *et al.* (2002). Transduction of hepatocytes after neonatal delivery of a Moloney murine leukemia virus based retroviral vector results in long-term expression of beta-glucuronidase in mucopolysaccharidosis VII dogs. *Mol Ther* **5**: 141–153.
36. Baum, C, Kustikova, O, Modlich, U, Li, Z and Fehse, B (2006). Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum Gene Ther* **17**: 253–263.
37. Vogler, C, Levy, B, Grubb, JH, Galvin, N, Tan, Y, Kakkis, E *et al.* (2005). Overcoming the blood-brain barrier with high-dose enzyme replacement therapy in murine mucopolysaccharidosis VII. *Proc Natl Acad Sci USA* **102**: 14777–14782.
38. Ma, X, Liu, Y, Tittiger, M, Hennig, A, Kovacs, A, Popelka, S *et al.* (2007). Improvements in mucopolysaccharidosis I mice after adult retroviral vector-mediated gene therapy with immunomodulation. *Mol Ther* **15**: 889–902.
39. Ciron, C, Desmaris, N, Colle, MA, Raoul, S, Joussemet, B, Verot, L *et al.* (2006). Gene therapy of the brain in the dog model of Hurler's syndrome. *Ann Neurol* **60**: 204–213.
40. Ponder, KP, Melniczek, JR, Xu, L, Weil, MA, O'Malley, TM, O'Donnell, PA *et al.* (2002). Therapeutic neonatal hepatic gene therapy in mucopolysaccharidosis VII dogs. *Proc Natl Acad Sci USA* **99**: 13102–13107.
41. Mango, RL, Xu, L, Sands, MS, Vogler, C, Seiler, G, Schwarz, T *et al.* (2004). Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. *Mol Genet Metab* **82**: 4–19.
42. Sleeper, MM, Fornasari, B, Ellinwood, NM, Weil, MA, Melniczek, J, O'Malley, TM *et al.* (2004). Gene therapy ameliorates cardiovascular disease in dogs with mucopolysaccharidosis VII. *Circulation* **110**: 815–820.
43. Meikle, PJ, Grasby, DJ, Dean, CJ, Lang, DL, Bockmann, M, Whittle, AM *et al.* (2006). Newborn screening for lysosomal storage disorders. *Mol Genet Metab* **88**: 307–314.
44. Wang, D, Eadala, B, Sadilek, M, Chamoles, NA, Turecek, F, Scott, CR *et al.* (2005). Tandem mass spectrometric analysis of dried blood spots for screening of mucopolysaccharidosis I in newborns. *Clin Chem* **51**: 898–900.