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Molecules and Cells

Characterization of a Novel Mucopolysaccharidosis Type II Mouse Model and Recombinant AAV2/8 Vector-Mediated Gene Therapy

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Mucopolysaccharidosis type II (MPS II; Hunter syndrome) is an X-linked inherited disorder caused by a deficiency of the enzyme iduronate-2-sulfatase (IDS), which results in the lysosomal accumulation of glycosaminoglycans (GAG) such as dermatan and heparan sulfate. Here, we report the generation of IDS knockout mice, a model of human MPS II, and an analysis of the resulting phenotype. We also evaluated the effect of gene therapy with a pseudotyped, recombinant adeno-associated virus 2/8 vector encoding the human IDS gene (rAAV-hIDS) in IDS-deficient mice. IDS activity and GAG levels were measured in serum and tissues after therapy. Gene therapy completely restored IDS activity in plasma and tissue of the knockout mice. The rescued enzymatic activity completely cleared the accumulated GAGs in all the tissues analyzed. This model can be used to explore the therapeutic potential of IDS replacement and other strategies for the treatment of MPS II. Additionally, AAV2/8 vectors have promising future clinical applications for the treatment of patients with MPS II.

INTRODUCTION

Mucopolysaccharidosis type II (MPS II), also known as Hunter syndrome [OMIM 309900], is a rare, X-linked lysosomal storage disorder caused by a deficiency of the enzyme iduronate-2-sulfatase (IDS; EC 3.1.6.13). This enzyme is involved in the lysosomal catabolism of glycosaminoglycans (GAG) such as heparan sulfate and dermatan sulfate. Lysosomal GAG accumulation damages cells and tissues with consequent organ dysfunction that manifests as various chronic and progressive patterns of clinical severity (Martin et al., 2008).

Animal models have contributed significantly to our understanding the pathogenesis of genetic diseases. Knockout mouse is a useful tool for disease model (Ko et al., 2003). Murine models of several of the MPS diseases have been developed and are being used for the evaluation of novel therapies (Birkenmeier et al., 1989; Clarke et al., 1997; Evers et al., 1996; Li et al., 1999; Tomatsu et al., 2003). A knockout (KO) mouse model of MPS II has been developed (Muenzer et al., 2002). Briefly, targeted disruption of the mouse IDS locus was achieved by deleting exon 4 and part of exon 5 using homologous recombination with a replacement vector containing the neomycin resistance gene.

Here, we report the generation of novel IDS KO mice and an analysis of the resulting phenotype. In addition, a pseudotyped, recombinant adeno-associated virus 2/8 vector encoding the human IDS gene (rAAV-hIDS) was administered intravenously to adult IDS-deficient mice to evaluate the effect of gene therapy in a mouse model of MPS II.

MATERIALS AND METHODS

Knockout mouse model

The KO mouse model of Hunter syndrome was generated by replacing a part of the IDS gene (1485 bp encompassing exon 2 and exon 3) with the neomycin resistance gene. First, we developed KO constructs containing the intron region between exon 1 and exon 2 of the IDS gene (3995 bp) for the left arm and a region from exon 4 to exon 7 (5447 bp) for the right arm (Fig. 1A). Polymerase chain reaction was performed to obtain DNA of both arms from a BAC clone (AC002315) containing the mouse X chromosome. Cloning to the pPNT vector (7348 bp) was performed using *Xho*I and *Eco*RI sites for the left and right arms, respectively. The linearized construct was transfected into embryonic stem cells, and the positive cell clones were selected by Southern blotting and microinjected into C57BL blastocysts. Targeted disruption of the mouse IDS locus at exon 2 and exon 3 was performed by homologous recombination with the neomycin resistance gene and the pPNT vector.

IDS knockout (IDS KO) mice were inbred and bred to a wild-type C57BL/6 strain. All offspring were genotyped using PCR of tail genomic DNA. The PCR primer set for IDS KO DNA was ATG ATT GAA CAA GAT GGA TTG CAC G (forward) and TCA GAA GAA CTC GTC AAG AAG GCG A (reverse) and that for wild-type IDS DNA was TCC TTC TGA TCA TTG TGG ATG A (forward) and TGA GCA AAG GCA TTC TGA AA (reverse). Offsprings generated wild-type male (IDS+/0) and fe-

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male (IDS+/+), hemizygous IDS KO male (IDS-/0), and heterozygous female (IDS+/-). Mice were maintained in specific pathogen-free conditions at the animal facility of the Samsung Biomedical Research Institute and Ewha Womans University School of Medicine.

Vector construction and production of rAAV

The AAV-2-based human IDS expression vector plasmid, pAAV2-EF-hIDS-WPRE, was constructed as described previously (Oh et al., 2004). To obtain the human IDS cDNA, total RNA was extracted from white blood cells of normal healthy male volunteer using QIAamp RNA blood kit (Qiagen, USA). Reverse transcription with oligo(dT) priming was performed to generate cDNAs from 1 μg total RNA using reverse transcriptase M-MLV (RNase H-) (Takara, Japan). To amplify human IDS cDNA, the primers (sense: 5′- ggt acc atg ccg cca ccc cgg acc, antisense: 5′- tct aga gg cat caa caa ctg gaa aag) were designed. (DNA amplification was carried out with Ex Taq DNA polymerase (Takara). RT-PCR products were cloned in pCR2.1 vector (Invitrogen, USA). Successful clones were confirmed by sequencing.

The full-length human IDS coding sequence was subcloned into pAAV2-EF-eGFP-WPRE-polyA by substitution with the eGFP sequence. The pAAV2-EF-hIDS-WPRE plasmid harbors the human elongation factor 1-a promoter (EF), woodchuck hepatitis virus posttranscriptional element (WPRE), and a bovine growth hormone poly(A) signal. The rep2/cap8 plasmid (p5E18-VD2/8, kindly provided by James M. Wilson) was used to package the expression vector. The pseudotyped rAAV2/8-hIDS was produced by triple plasmid transfection and purified by cesium chloride (Sigma-Aldrich, USA) density gradient ultracentrifugation. The rAAV2/8-hIDS genomic titer was determined using real-time quantitative PCR (ABI 7000; Applied Biosystems, USA), in which the signal from aliquots of the test material is compared with a standard signal generated using the linearized pAAV2-EF-hIDS-WPRE plasmid.

AAV vector administration to IDS KO mice

All mice were genotyped by PCR as described previously (Ohshima et al., 1997). Mice were fed an autoclaved diet and water ad libitum. Male IDS KO mice (n = 3) were injected with 1.0×10^{11} particles of rAAV2/8-hIDS in a volume of 200 μl via the tail vein at 20 weeks of age. Another three IDS KO and three wild type mice were not treated. All animals were cared for in accordance with the Animal Care Guidelines of the Ewha Womans University School of Medicine, Samsung Biomedical Research Institute, and the US National Institutes of Health.

IDS activity assay

Tissue samples were homogenized in phosphate-buffered saline (PBS) using a glass grinder and centrifuged at 20,000 × *g* for 30 min at 4°C. Serum and tissue protein concentrations were determined using the BioRad protein assay (BioRad, USA). The IDS activity in tissue extracts and serum samples was determined by the two-step fluorometric assay using the substrate 4-methylumbelliferyl-α-L-iduronidase-2-sulphate (Rotterdam, The Netherlands). The IDS assay was performed as described previously (Voznyi et al., 2001).

Quantitative analysis of GAG accumulation

Tissue samples were homogenized in PBS and centrifuged at 20,000 × *g* for 30 min. GAG concentrations in serum and tissue extracts were quantified by a colorimetric assay using Alcian blue dye (Kamiya Biomedical Co., USA) and measuring absorbance at 620 nm. GAG concentrations were determined using a chondroitin 6-sulphate standard curve.

Toluidine blue staining

After perfusing the animals with PBS, the brains were collected and fixed with 4% PFA for 24 h at 4°C. The tissue was then incubated overnight in 30% sucrose at 4°C. Finally, the tissue was embedded in OCT compound (Sakura Finetek, USA) and frozen in a bath of dry ice and ethanol. The brains were sliced into 8-μm-thick serial sections using a microtome. The sections were stained with 0.1% toluidine blue.

Western blotting

Tissue samples were homogenized in PRO-PREP solution (Intron Biotechnology, Korea) using a glass grinder, and protein concentrations were determined using the BioRad protein assay (BioRad, USA). Protein electrophoresis was performed on a 10% polyacrylamide gel, and the protein was blotted onto a PVDF membrane (Millipore, USA). The membrane was hybridized with a 1:250 dilution of human IDS monoclonal antibody (R&D Systems, USA) and subsequently with a horseradish peroxidase-conjugated anti-mouse antibody. The signals were visualized using an ECL detection system (GE Healthcare, UK).

Statistical analysis

Statistically significant differences between groups were determined using Student's *t* test. Data are presented as mean ± standard deviation. Null hypothesis probabilities of < 0.05 were considered statistically significant.

RESULTS

Generation of the KO mouse model of Hunter syndrome

The IDS gene was inactivated by deletion of exon 2 and exon 3 (Fig. 1A), and Southern blot analysis of genomic DNA obtained from offspring tail vein blood discriminated wild-type (+/+), hemizygous male (+/0), heterozygous female (+/-), and homozygous female (-/-) mice (Fig. 1B). Male mice were either hemizygous or wild-type because the IDS gene is located on the X chromosome. Female mice were wild-type (+/+), and homozygous females were uniformly hemizygous.

Phenotypic analysis of IDS KO mice

At birth, no morphologic differences were seen in wild-type and IDS KO offspring. We did not discern the genotype of the offspring because DNA sampling from the tail vein was not carried out until the pups reached 4 weeks of age. Differences in behavior or the external appearance of wild-type and IDS KO offspring were assessed 4 to 5 weeks after birth. The first morphological changes in IDS KO male offspring were coarse facial features and sporadic alopecia, which were observed at 5 to 6 weeks of age, followed by limitations of the hind limb joint when the tail was pulled back, which were observed at 7 to 8 weeks. The external facial appearances of male IDS KO mice in the affected male were different from those of wild-type littermates at 5 to 6 weeks of age. The face was relatively larger and the snout was more prominent in IDS KO males when observed from the lateral side (Fig. 2A). The articulation of the foot in the knockout mice was restricted in the forelimb and resembled a clawed hand. From 4 to 11 weeks of age, IDS KO mice were heavier than the wild-type littermates, but the latter became heavier after 12 weeks (data not shown). Around 25 to 30 weeks of age, the physical activity of KO mice declined, and most had died before 1 year of age.

Urinary GAG excretion was measured beginning at postnatal

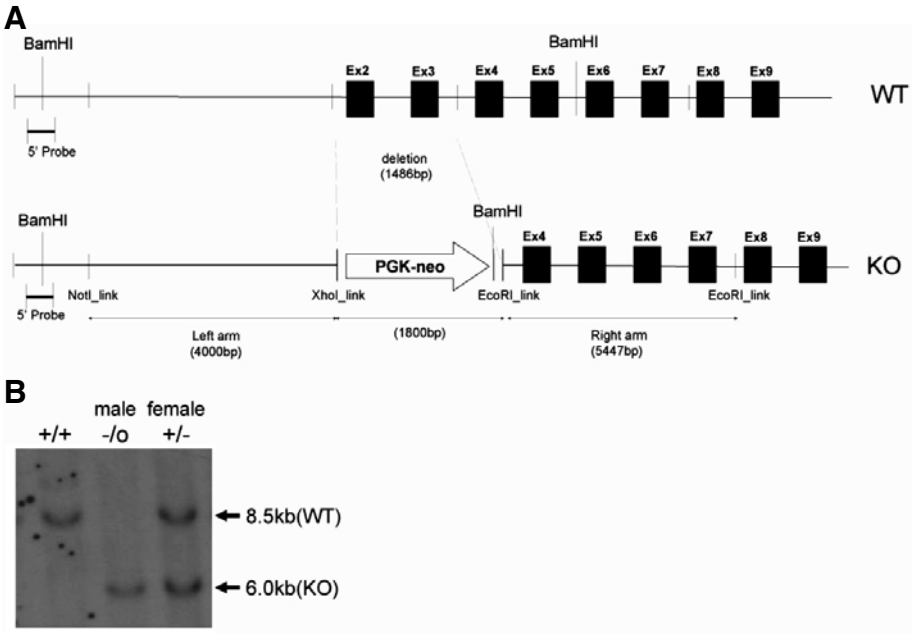

Fig. 1. Targeting construct and screening by Southern blotting. (A) Targeted IDS gene. (B) Southern blot analysis of genomic DNA obtained from offspring tail of wild-type male (+/+), hemizygous male (-/O) and heterozygous female (+/-).

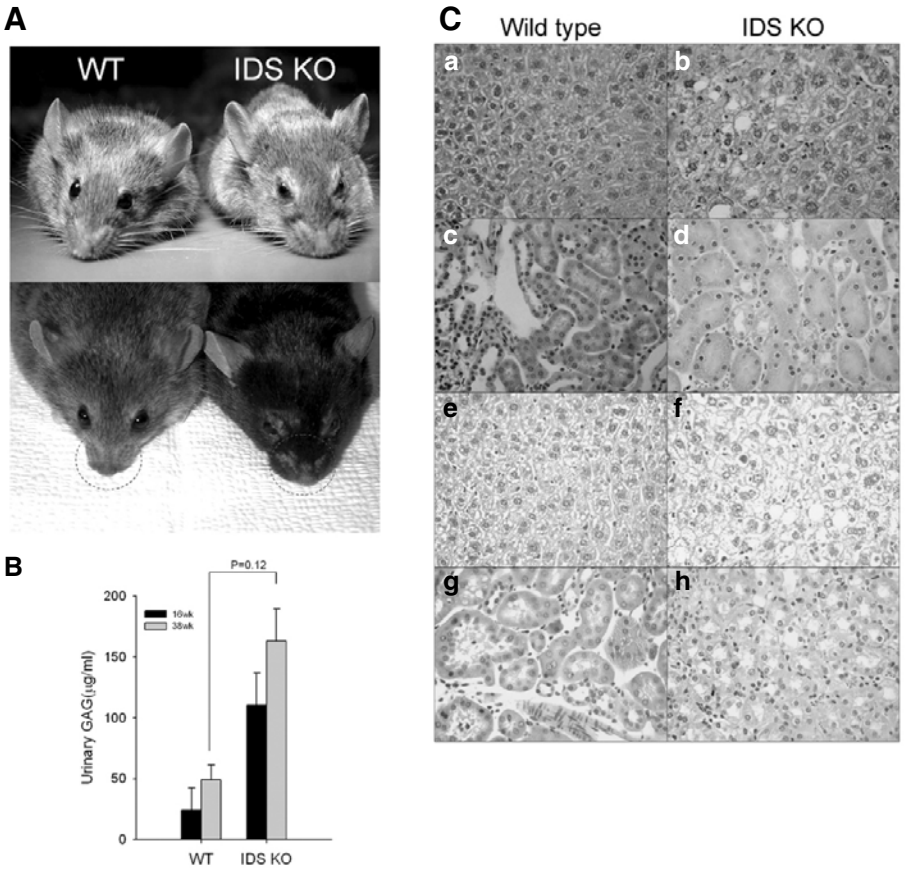

Fig. 2. Characterization of IDS KO mouse. (A) Phenotype of wild-type and IDS KO mouse. Circles represent the same area to compare the facial size (B) Measurement of urinary GAG in 16 weeks mouse and 38 weeks (C) Histological analysis of wild-type (a, c, e, and g) and IDS KO (b, d, f, and h). The tissues from (a, b, e, and f) came from liver and (c, d, g, and h) from kidney respectively. H&E staining (a-d) and Alcian blue staining (e-h).

week 4, and it was greater in IDS KO mice. The increased GAG excretion was more prominent at 16 and 38 weeks of age relative to excretion in wild-type littermates of the same age (Fig. 2B). Extracts of the brain, liver, spleen, and kidney as well as serum from 16-week-old IDS KO mice showed no IDS activity.

Histological examination of the IDS KO mice revealed many foamy cells, which is a characteristic of lysosomal storage disease. These foamy cells were found in the liver, kidney, lung, heart, brain, and lymph node extracts in varying amounts (Fig. 2C). IDS KO mice had elevated GAG content in a variety of tissues, including the liver, lung, heart, brain, spleen, kidney, and skin, as revealed by intense Alcian blue stains.

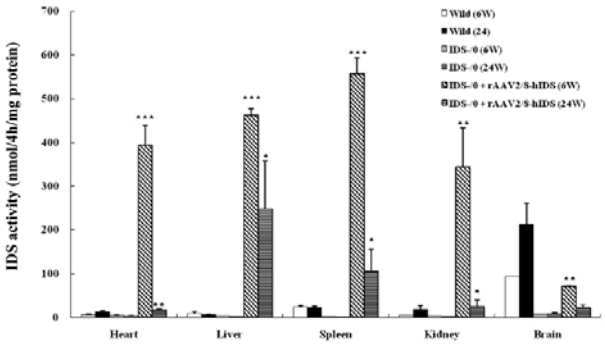

Fig. 3. IDS activity in IDS KO mice after rAAV2/8-hIDS administration via the tail vein. Organs were obtained from each group of mice killed 6 weeks and 24 weeks after r AAV2/8 vector administration. The enzyme activity is expressed as means ± SD. *, P < 0.05, **, P < 0.01, and ***, P < 0.001 compared to IDS KO mice (n = 3).

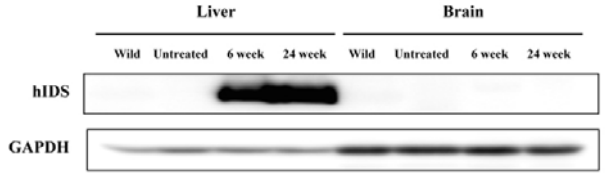

Fig. 4. Western blot analysis of human IDS (hIDS) in tissues (liver and brain) 6 and 24 weeks after intravenous administration of rAAV2/8-hIDS to IDS KO mice.

IDS activity in the tissues of IDS KO mice by rAAV2/8 hIDS

Adult IDS KO mice (age 20 weeks; n = 3) received 1.0×10^{11} particles of AAV2/8 hIDS in a volume of 200 μl via the tail vein. The mice were sacrificed either 6 or 24 weeks after treatment (n = 3 for both time periods). In parallel, untreated IDS KO (n = 3) and wild-type mice (n = 3) were sacrificed as controls. IDS activity and GAG concentrations in the heart, liver, spleen, kidney, brain, and serum were also analyzed 6 and 24 weeks following vector administration.

IDS activity in liver samples from the rAAV2/8-hIDS-treated IDS KO mice 6 weeks after treatment was 50.5 ± 1.6 times higher than that measured in the wild-type mice (Fig. 3). Serum IDS activity was 62.1 ± 10.2 and 60.5 ± 13.7 times higher at 6 and 24 weeks, respectively, in treated mice than in the untreated wild-type mice (data not shown). Similar or higher IDS activity was seen in treated mice compared to wild-type mice in the other tissues (heart, spleen, kidney, and brain).

Expression of human IDS protein was determined in liver and brain tissue of treated mice by Western blotting. A robust signal against a monoclonal anti-human IDS was seen at 6 and 24 weeks in liver tissue (Fig. 4), but was not detected in the brain, perhaps lower than the detection limit for IDS.

Clearance of accumulated lysosomal GAG in treated IDS KO mice

GAG concentrations in the different tissues of treated IDS KO mice (n = 3) were determined 6 and 24 weeks after gene therapy. Tissue GAG concentrations were also determined in untreated IDS KO (n = 3), and wild-type (n = 3) adult mice. All treated mice had lower GAG accumulation than did the control mice (Fig. 5). GAG concentrations in the treated mice returned to normal during the therapy.

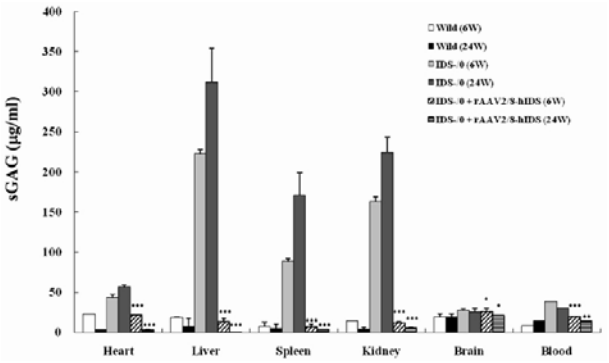

Fig. 5. GAG assay of IDS KO mice after rAAV2/8-hIDS administration via the tail vein. Data are expressed as means ± SD. *, P < 0.05, **, P < 0.01, ***, P < 0.001 compared to IDS KO mice (n = 3).

Histological analysis

Histological analysis of brain sections of the treated IDS KO mice was performed 24 weeks after treatment and age-matched, untreated IDS KO mice using toluidine blue staining. As shown in Fig. 6, treatment reduced cellular vacuolization.

DISCUSSION

We generated an IDS KO mouse model and evaluated the effect of gene therapy with a pseudotyped rAAV 2/8 vector encoding the human IDS gene. Our MPS II murine model showed some similar pathology to that seen in Hunter syndrome patient and is similar to a previously described mouse model (Garcia et al., 2007). Because the previously reported KO mouse was not available with us, we attempted to compare the reported findings with our own experience. We could distinguish wild-type and IDS KO mice at 5 to 6 weeks of age, whereas in the previous report, morphologic abnormalities associated with IDS KO were apparent by 10 weeks of age (Garcia et al., 2007). However, the appearance of IDS KO mice was similar to that of the IDS KO mice investigated in the previous study: coarse fur, sporadic alopecia, and gibbous deformities in the hind limb articulations that impeded joint mobility. The histological features and the appearance of urinary GAG were similar to those reported previously, but accumulation of GAG in the liver was observed as early as 12 weeks. The life span of our IDS KO mice and that of the previous study was approximately 1 year.

Our mouse model containing a deletion in front of the IDS gene (exon 2 and exon 3) is likely to severely diminish IDS expression. Therefore, the IDS KO mouse model exhibited disease symptoms rapidly: tissue GAG content, especially in the liver, in our model was 18 times at 12 weeks but 10 times at 40 weeks in the previous model (Garcia et al., 2007). These findings suggest that our IDS KO mouse model can be useful in pre-clinical trials and for showing changes in symptoms after enzyme treatment.

Currently, the treatment of MPS II is limited to enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). The usefulness of the former is limited because it is expensive and the enzyme has brief blood and intracellular half-lives, which necessitates weekly administration (Brooks et al., 2003). BMT dose not improve the neuropsychological function of patients with severe forms of MPS II, including mental impairment (Coppa et al., 1995; Li et al., 1996; Malatack et al., 2003; Peters and Krivit, 2000; Vellodi et al., 1999). Gene trans-

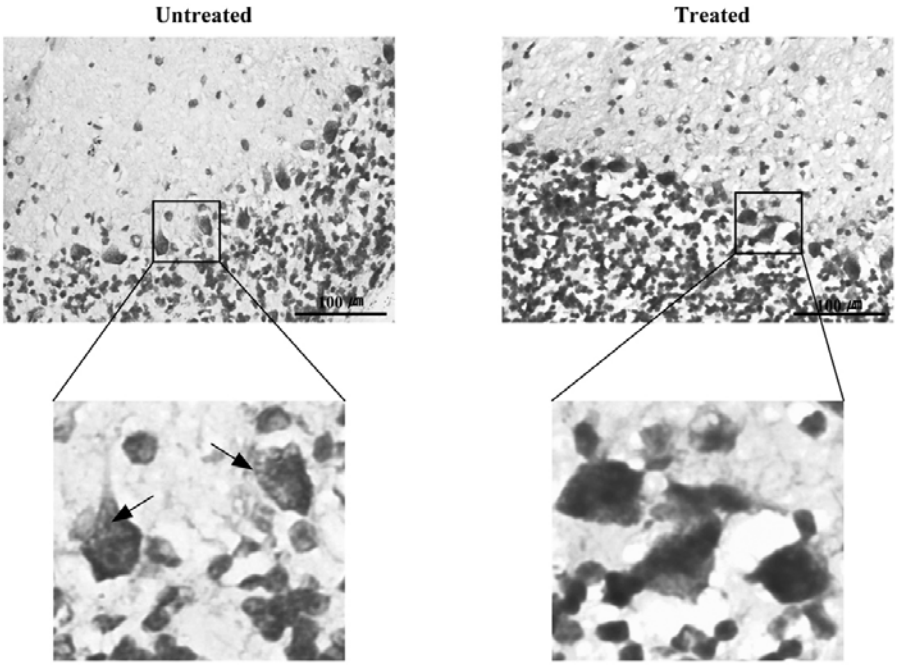

Fig. 6. Neuropathological analysis. Toluidine blue staining of cerebellar sections from untreated mice and treated IDS KO mice (24 weeks after treatment); magnification, 200×. Vacuolization and morphological alterations are shown as indicated by arrows (bottom square). A higher magnification was 1000×. Scale bar is 100 μm.

fer technology has also been used to deliver a recombinant IDS-coding sequence through viral vectors (Braun et al., 1993; Daniele et al., 2002; Di Francesco et al., 1997; Whitley et al., 1996). However, this too has limitations. Gene transfer using retroviruses may be susceptible to insertional mutagenesis (Hacein-Bey-Abina et al., 2003); furthermore, adenovirus-mediated gene delivery may be associated with immunogenicity and an inflammatory response (Liu and Muruve, 2003; Muruve, 2004; Tomanin and Scarpa, 2004). We used the pseudotyped rAAV2/8 vector as a gene-delivery vehicle, and this approach completely restored IDS activity in plasma and tissues. This rescue of enzymatic activity fully cleared accumulated GAGs from all the tissues analyzed.

Of considerable interest is the effect of gene therapy on brain IDS. In our model, gene therapy increased IDS activity and decreased GAG accumulation in this organ. Moreover, it improved histological features. No definitive description of the change in brain pathology during enzyme replacement exists. Therefore, this improvement in the histology of the brain may be one advantage of gene therapy, and one that may be a promising avenue of future research.

In conclusion, AAV2/8 vectors may have promising clinical applications in the treatment of patients with MPS II.

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