Liver Production of Sulfamidase Reverses Peripheral and Ameliorates CNS Pathology in Mucopolysaccharidosis IIIA Mice

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Mucopolysaccharidosis type IIIA (MPSIIIA) is an inherited lysosomal storage disease caused by deficiency of sulfamidase, resulting in accumulation of the glycosaminoglycan (GAG) heparan sulfate. It is characterized by severe progressive neurodegeneration, together with somatic alterations, which lead to death during adolescence. Here, we tested the ability of adeno-associated virus (AAV) vector-mediated genetic modification of either skeletal muscle or liver to revert the already established disease phenotype of 2-month-old MPSIIIA males and females. Intramuscular administration of AAV-Sulfamidase failed to achieve significant therapeutic benefit in either gender. In contrast, AAV8-mediated liver-directed gene transfer achieved high and sustained levels of circulating active sulfamidase, which reached normal levels in females and was fourfold higher in males, and completely corrected lysosomal GAG accumulation in most somatic tissues. Remarkably, a 50% reduction of GAG accumulation was achieved throughout the entire brain of males, which correlated with a partial improvement of the pathology of cerebellum and cortex. Liver-directed gene transfer expanded the lifespan of MPSIIIA males, underscoring the importance of reaching supraphysiological plasma levels of enzyme for maximal therapeutic benefit. These results show how liver-directed gene transfer can reverse somatic and ameliorate neurological pathology in MPSIIIA.

Received 29 June 2010; accepted 16 September 2011; advance online publication 18 October 2011. doi:10.1038/mt.2011.220

INTRODUCTION

Type III mucopolysaccharidoses (MPSIII), or Sanfilippo syndrome, are lysosomal storage diseases (LSD) caused by the deficiency in one of the enzymes involved in the degradation of heparan sulfate, leading to the pathological accumulation of this substrate. MPSIII is classified into four subtypes depending on the enzyme deficiency. Subtype IIIA is caused by the loss of sulfamidase activity. Sulfamidase is a sulfatase that releases the sulfate linked to the amino group of the terminal glucosamine of heparan sulfate, and its deficiency has been reported to be the most severe form of MPSIII, with the earliest disease onset and shortest survival.[1] Symptoms of MPSIIIA appear in the first years of life, and are characterized by severe neurodegeneration that leads to deep mental retardation, aggressiveness, hyperactivity, and sleep alterations. Patients progressively lose the ability to speak, swallow, and basic motor coordination. MPSIIIA patients also suffer non-neurological alterations, including hepato- and splenomegaly, skeletal, and joint malformations, as well as frequent diarrhea and respiratory tract infections. Affected subjects usually die during adolescence.[2] A mouse model of MPSIIIA exists, arising from a spontaneous missense mutation in the sulfamidase gene that dramatically reduces sulfamidase activity to 3% of wild-type (WT).[3,4] MPSIIIA mice have been reported to closely reproduce the human disease, presenting with hepato-splenomegalia, neurodegeneration, neuroinflammation, and shortened lifespan.[4–6] Likely due to the residual enzymatic activity MPSIIIA mice have a milder phenotype than other MPSIII mouse models.[7]

Currently there is no cure for MPSIIIA and, therefore, existing treatments are aimed at controlling the symptoms in order to improve the quality of life of patients. Many LSDs can be treated by bone marrow transplantation or enzyme replacement therapy (ERT). Both approaches rely on the endocytosis of lysosomal enzymes from extracellular medium and their targeting to lysosomes via the mannose-6-phosphate receptor (M6PR) present at the cell membrane. Nevertheless, bone marrow transplantation is inefficient in the treatment of MPSIII patients.[8] ERT has been extensively proven to be effective in counteracting the non-neurological accumulation in other LSDs, including MPSI, II and VI.[9] In addition to its high cost, ERT does not result in correction or preservation of neuronal function due to the insufficient delivery of the exogenously provided enzyme through the blood-brain barrier (BBB),[10] although it could be overcome by using very high doses.[11] Intracerebral and intra-cerebrospinal fluid delivery of the enzyme have also been shown to ameliorate central nervous system pathology in MPSIIIA mice.[12,13] However, this approach is

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highly invasive due to the short half-life of the protein and the consequent need for repeated injections, which could increase the risk of brain damage and/or infection.

Given the limitations of current therapeutic options for MPSIII, alternative approaches are needed. Gene transfer could provide the means to achieve sustained production of the missing enzyme from a single administration. Adeno-associated virus (AAV) vectors have shown promising results in several *in vivo* applications.[14] AAV vectors have been shown to efficiently transduce postmitotic cells, including CNS or liver, and several preclinical and clinical studies demonstrated the potential of AAV to drive multi-year expression of therapeutic transgenes for a variety of diseases.[15] The use of an AAV5 vector co-expressing sulfamidase and the sulfatase activator Sulfatase Modifying Factor-1 (SUMF1) in lateral ventricles showed correction of many neurological and behavioral alterations in newborn MPSIIIA mice.[16] However, MPS patients are rarely diagnosed at birth, and thus, therapeutic interventions should aim at correcting the disease after onset. Intravenous delivery of a lentiviral vector expressing sulfamidase in adult MPSIIIA mice resulted in little amelioration of the CNS phenotype. The efficacy of the approach was likely hampered by the relatively poor transduction performance of these vectors *in vivo*.[17] Thus, the use of viral vectors with higher transduction performance *in vivo*, such as AAV vectors, may provide higher circulating levels of sulfamidase and potentially ameliorate or correct the neurological pathology.

Here we tested the therapeutic efficacy of a new gene transfer approach for MPSIIIA based on the use of sulfamidase-expressing AAV1 and AAV8 vectors. These vectors were administered to adult MPSIIIA mice by intramuscular (i.m.) injection, to target the skeletal muscle, or by intravenous (i.v.) injection, to target the liver. Our results provide evidence of the therapeutic potential of AAV-mediated gene transfer of murine sulfamidase to the liver of adult MPSIIIA mice, a disease model closely resembling the clinical setting.

RESULTS

Genetic modification of skeletal muscle to produce sulfamidase results in poor secretion of the enzyme into the circulation

At 2 months of age, MPSIIIA mice displayed higher levels of accumulated glycosaminoglycan (GAG) in all somatic tissues and in the brain, compared to healthy wild-type mice (**Supplementary Figure S1a,b** and data not shown). Immunostaining for lysosomal-associated membrane protein 1 (LAMP1), a lysosomal marker, revealed that the liver of 2-month-old MPSIIIA mice had enlarged lysosomal compartment compared to WT (**Supplementary Figure S1c**). These data indicated that pathological alterations characteristic of MPSIIIA already existed at this age.

To examine whether MPSIIIA pathology could be reverted in adult mice, 2-month-old male and female MPSIIIA mice were intramuscularly injected with 10^{12} vector genomes of either AAV1-CAG-murineSulfamidase-WPRE (i.m.-AAV1CAG) or AAV8- CAG-murineSulfamidase-WPRE (i.m.-AAV8CAG) (**Supplementary Figure S2a**). Eight months after the AAV vector administration, *i.e.*, at 10 months of age, exogenous sulfamidase messenger RNA (mRNA) transcripts were detected in the skeletal muscle of all animals injected. Muscles of AAV1-treated animals showed higher levels of sulfamidase expression than AAV8-treated animals (**Supplementary Figure S2b**). Sulfamidase activity in the skeletal muscle of i.m.-AAV8CAG-treated animals reached the levels of healthy muscle (**Supplementary Figure S2c**). In contrast, in i.m.-AAV1CAG-treated animals it rose 50-fold above the levels of WT. Skeletal muscle of AAV1-treated females displayed higher levels of sulfamidase activity than males (**Supplementary Figure S2c**). Exogenous sulfamidase mRNA transcripts were also detected in the liver of intramuscularly treated animals, suggesting a leakage of the vector to systemic circulation (**Supplementary Figure S2d**). Consistently, low levels of sulfamidase activity were detected in the liver of intramuscularly treated mice compared to nontreated MPSIIIA mice (**Supplementary Figure S2e**). Sulfamidase activity in serum reached about 5% of normal levels in i.m.-AAV1CAG-treated animals, and up to 10–20% in the i.m.-AAV8CAG-treated group (**Supplementary Figure S2f**). The lack of correlation between the activity of sulfamidase in skeletal muscle and serum levels of sulfamidase suggests that the skeletal muscle is unable to secrete significant amounts of the enzyme into the circulation, and the liver is probably the main source of the circulating sulfamidase.

Genetic modification of the liver results in high sulfamidase activity in serum

We next examined whether AAV-mediated liver transduction could result in higher production and secretion of sulfamidase than skeletal muscle transduction. Two-month-old MPSIIIA mice were treated intravenously with 10^{12} vector genomes of either an AAV8-CAG-murineSulfamidase-WPRE (i.v.-AAV8CAG) vector, or a vector in which sulfamidase expression was driven by the hepatocyte-specific human α-antitrypsin (hAAT) promoter,[18] the AAV8-hAAT-murineSulfamidase (i.v.-AAV8hAAT) (**Figure 1a**). **Supplementary Table S1** shows vector biodistribution in somatic tissues. Eight months after administration, mice treated with i.v.-AAV8hAAT showed higher levels of sulfamidase mRNA expression in liver than the i.v.-AAV8CAG-treated mice (**Figure 1b**). Higher sulfamidase mRNA levels were found in males than females in both experimental cohorts. Sulfamidase activity was increased in the liver of all treated animals compared to nontreated MPSIIIA mice. In agreement with mRNA levels, i.v.-AAV8hAAT-treated mice had higher levels of sulfamidase activity (three to fourfold) than the i.v.-AAV8CAG-treated mice (**Figure 1c**), and sulfamidase activity was fourfold higher in the liver of males than females. This was parallel to an increase in serum sulfamidase activity in animals transduced with either vector (five to sixfold higher for i.v.-AAV8hAAT group than for i.v.-AAV8CAG), suggesting that the liver efficiently secreted sulfamidase into the bloodstream (**Figure 1d**).

Both muscle- and liver-directed gene transfer correct hepatic GAG accumulation

To determine whether increased sulfamidase activity resulted in normalization of the hepatic pathology, GAG accumulation was measured in liver extracts of mice killed 8 months after vector administration. Untreated MPSIIIA males displayed higher levels of GAG accumulation in the liver compared with nontreated MPSIIIA females at 10 months of age (**Figure 1e**). All AAV-treated animals, both i.m. and i.v., showed a marked reduction in

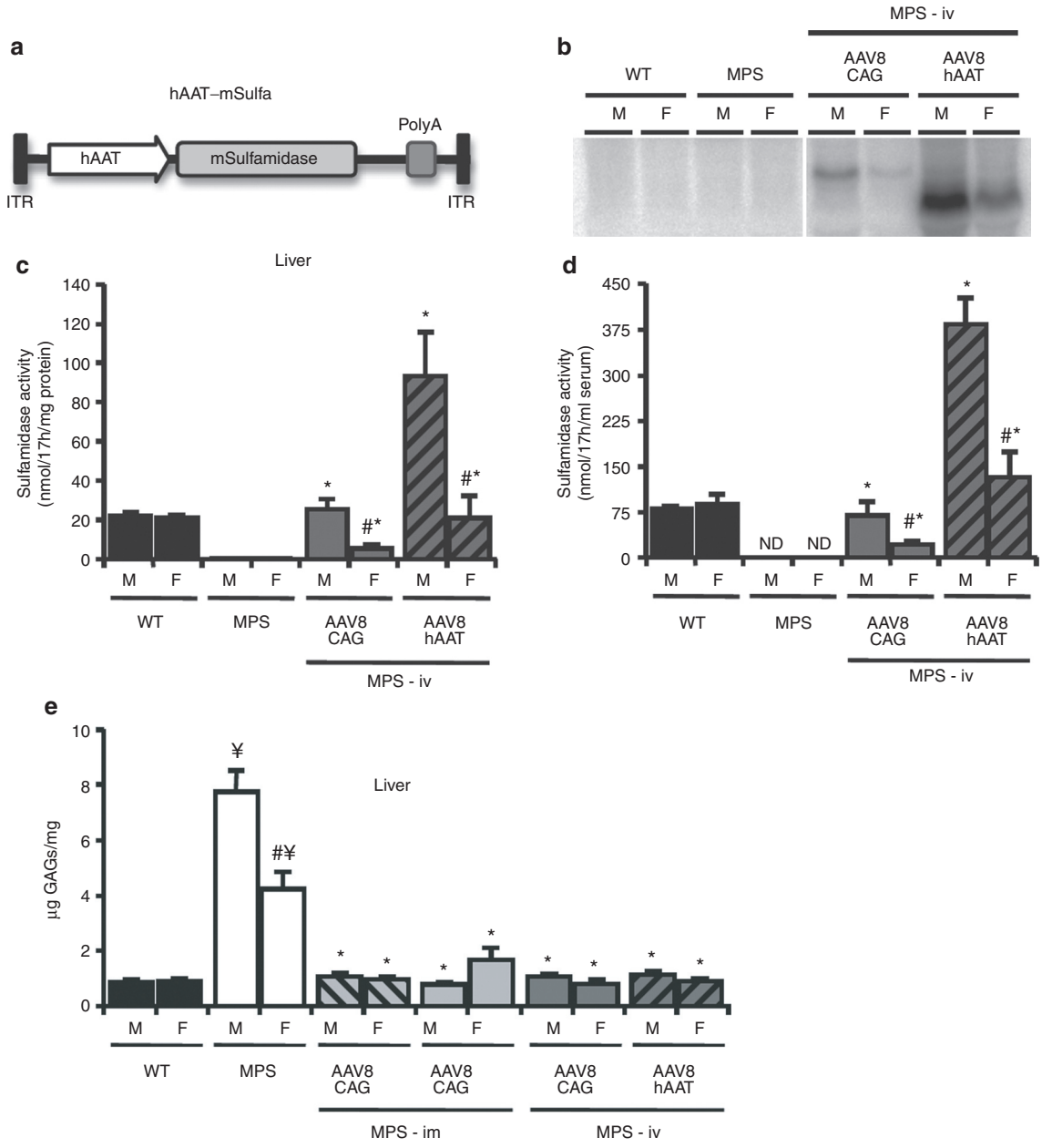

Figure 1 Genetic modification of the liver to produce sulfamidase and correction of liver GAG storage. Two-month-old MPSIIIA mice were intravenously treated with either AAV8CAG-murineSulfamidase or AAV8-hAAT-murineSulfamidase vectors and analyzed 8 months later. (**a**) Genome of AAV8-hAAT-murineSulfamidase vector. (**b**) Sulfamidase expression in liver analyzed by Northern blot. A representative blot is shown. The difference in size between the sulfamidase mRNAs derived from the AAV8CAG and AAV8-hAAT constructs is due to the presence of the WPRE sequence (~600 bp) in the former. (**c**) Sulfamidase activity in the liver measured using a fluorogenic substrate. (**d**) Sulfamidase activity in serum. (**e**) GAG storage in the liver of i.m. and i.v.-treated mice. GAG content was determined as indicated in materials and methods, and normalized to tissue weight. Values in **c**, **d**, and **e** are means ± SEM of 4–8 mice per group. $^{¥}P < 0.05$ versus control, $^{\#}P < 0.05$ versus males, $^{*}P < 0.05$ versus untreated MPS. F, females; GAG, glycosaminoglycan; hAAT, human α-antitrypsin; ITR, inverted terminal repeat; M, males; MPSIIIA, mucopolysaccharidosis type IIIA; mRNA, messenger RNA; ND: not detected.

the GAG content in the liver to levels similar to those of healthy WT animals (**Figure 1e**). This was associated with a clear reduction in the number and/or size of lysosomes in the liver of all treated animals as revealed by immunohistochemical staining for LAMP1 (**Supplementary Figure S3**). By electron microscopy, it was observed that male MPSIIIA mice had distended lysosomes in both hepatocytes and Kupffer cells, whereas in females, hepatocytes had hardly any storage vacuoles and Kupffer cells were filled with large lysosomes (**Figures 2a,b**). i.v.-AAV8CAG treatment completely normalized the ultrastructure of hepatocytes and Kupffer cells in both genders.

Both intravenously delivered AAV8 vectors completely normalized the hepatomegaly in both genders (**Supplementary Figure S4a**). Nontreated MPSIIIA males displayed a marked increase in hepatocyte area compared to those of WT males, whereas no difference was observed in females. Treated males had

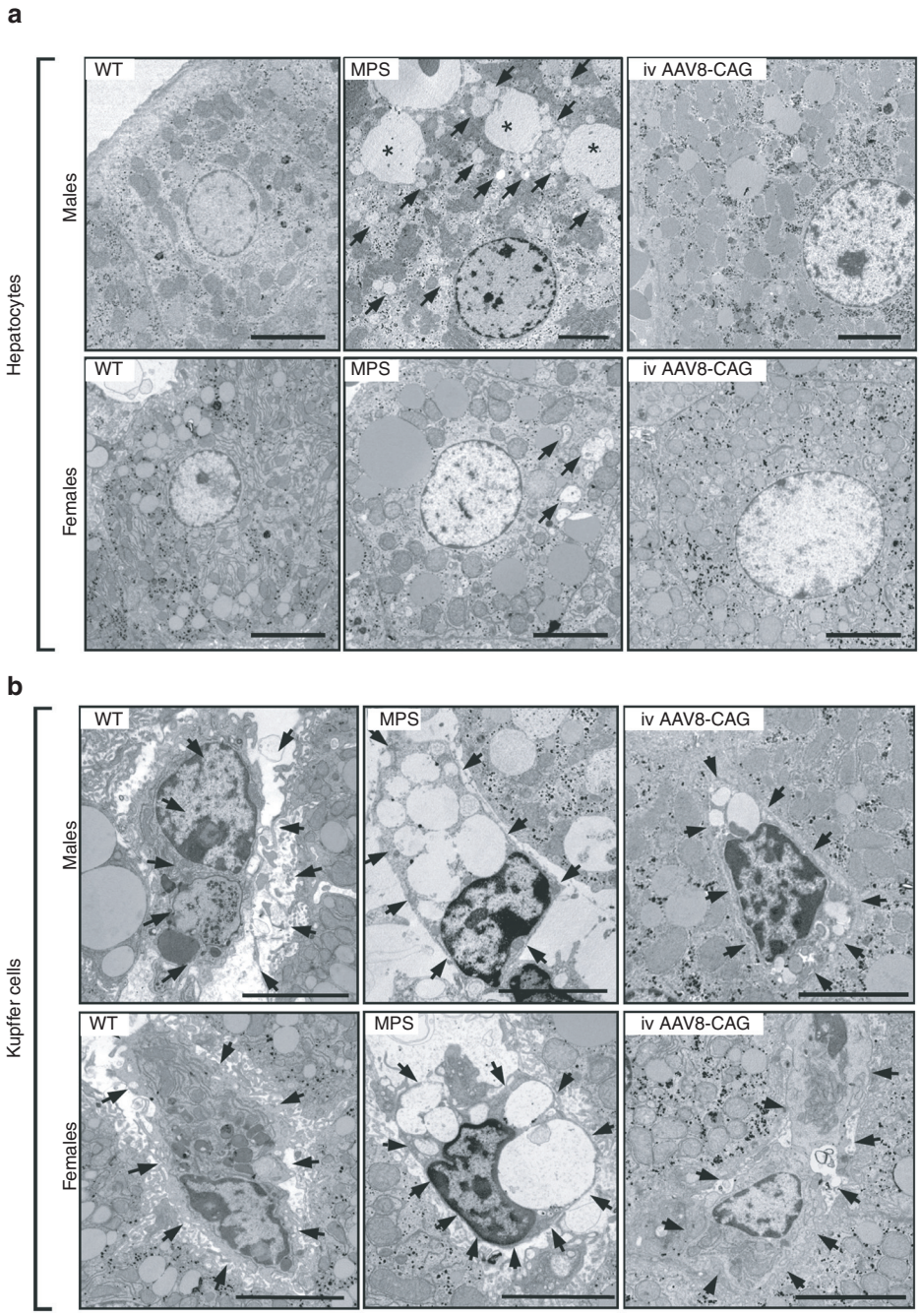

Figure 2 Correction of the ultrastructure of the liver. (**a**) Transmission electron microscopy of liver sections from healthy WT animals, untreated MPSIIIA animals, or i.m. and i.v. AAV-treated MPSIIIA mice. Hepatocytes of untreated MPSIIIA males contained many large (asterisks) and small (arrows) storage vacuoles, whereas hepatocytes from females had hardly any vacuoles. After intravenous administration of AAV8CAG vectors the storage vacuoles disappeared in both genders. (**b**) Kupffer cells (delimited by arrows) in livers of nontreated MPSIIIA mice were filled with several large electron-lucent vacuoles in both genders; vacuoles were strikingly reduced in i.v.-AAV8CAG treated mice. Bars: 5 µm. AAV, adeno-associated virus; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

reduced hepatocyte area, nearly reaching the size of healthy WTs (**Supplementary Figure S3b** and data not shown).

Genetic modification of the liver efficiently corrects somatic pathology

All treatments led to a significant reduction of the GAG accumulation in almost every somatic tissue analyzed (**Figure 3a–g** and **Supplementary Figure S5a**). Whereas i.m. and i.v.-AAV8CAG treatments only mediated a partial recovery of the GAG levels in some tissues (such as heart, lungs, kidney, and spleen), i.v.-AAV8hAAT treatment was able to completely revert GAG accumulation to the levels of healthy WT mice in all tissues analyzed. Urine GAG excretion was also normalized in all treatment cohorts (**Figure 3h**).

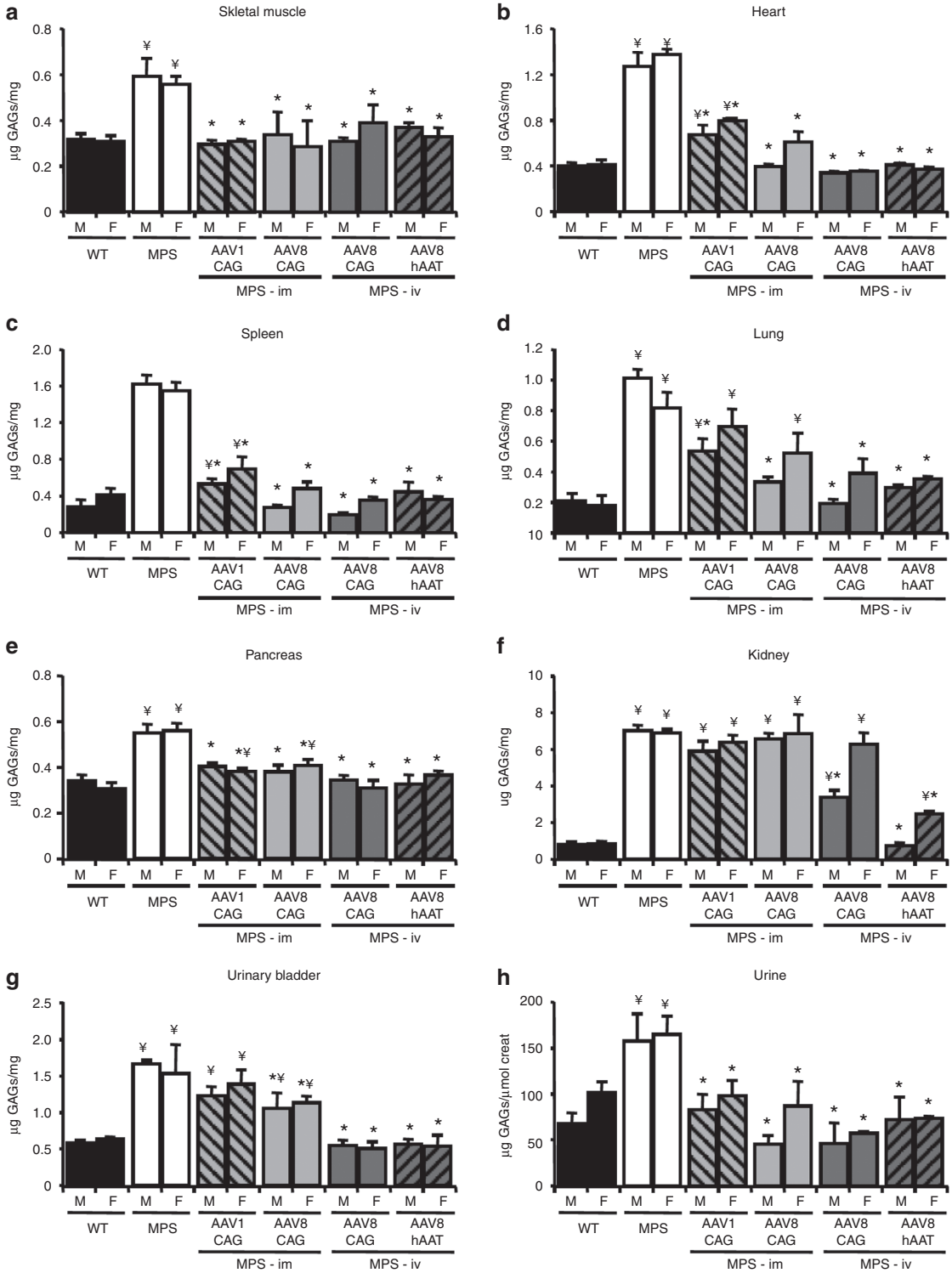

Figure 3 GAG storage in somatic tissues and GAG excretion by urine. (**a–g**) GAG storage in (**a**) skeletal muscle, (**b**) heart, (**c**) spleen, (**d**) lung, (**e**) pancreas, (**f**) kidney, and (**g**) urinary bladder. (**h**) GAG excretion in urine. GAG content was normalized to tissue weight or creatinine concentration. Results in **a–g** are means ± SEM of 4–8 mice per group. ¥$P < 0.05$ versus control, *$P < 0.05$ versus untreated MPS. F, females; GAG, glycosaminoglycan; M: males; MPS, mucopolysaccharides.

In agreement with the correction of GAG storage in somatic tissues of i.v.-AAV8hAAT treated mice, LAMP1 staining for lysosomes was normalized (**Figure 4** and **Supplementary Figure S5b**). Intense LAMP1-positive staining was revealed in reticuloendotelial cells within the red pulp of the spleen in nontreated MPSIIIA mice, but not in i.v.-AAV8hAAT treated mice (**Figure 4a–c**). Many large LAMP1-positive cells were also found infiltrating several tissues of untreated mice, such as lungs, exocrine pancreas,

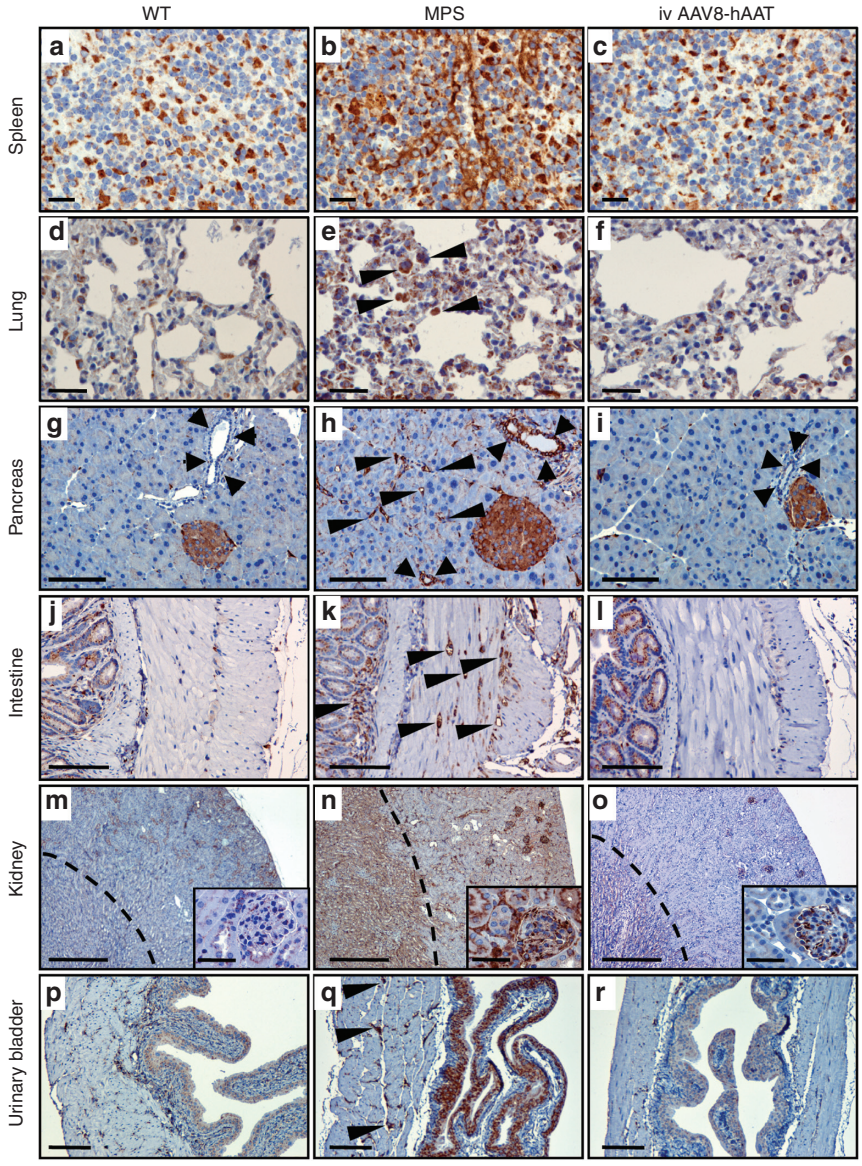

Figure 4 LAMP1 immunostaining in somatic tissues. LAMP1 immunohistochemistry of somatic tissues of healthy WT, untreated MPSIIIA, and i.v.-AAV8-hAAT treated MPSIIIA mice. (**a–c**) spleen, (**d–f**) lung, (**g–i**) pancreas, (**j–l**) intestine, (**m–o**) kidney, and (**p–r**) urinary bladder. Long arrowheads in **e, h, k**, and **q** mark the location of infiltrating LAMP1-positive cells. Short arrowheads in pancreas (**g–i**) mark the location of pancreatic ducts. Insets in kidney images (**m–o**) show a close-up of renal glomeruli. Bars are 25 µm for spleen (**a–c**) and lung (**d–f**), 100 µm for pancreas (**g–i**), intestine (**j–l**), and urinary bladder (**p–r**), and 500 µm for kidney (**m–o**; 20 µm for insets). hAAT, human α-antitrypsin; LAMP1, lysosomal-associated membrane protein 1; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

muscular layers and lamina propria of the colon, muscular layer of the urinary bladder, testicular interstitium and heart, and skeletal muscle (**Figures 4e,h,k,q** and **Supplementary Figure S5b** and data not shown). Double immunostaining for LAMP1 and Mac2 identified infiltrating cells as macrophages (**Supplementary Figure S6**). LAMP1-positive macrophages were absent in the tissues from i.v.-AAV8hAAT-treated mice (**Figure 4f,i,l,r** and **Supplementary Figure S5b**). In addition, untreated mice showed very intense LAMP1 staining in epithelial layers of some tissues, such as pancreatic ducts (**Figure 4h**), terminal bronchioles in the lungs (data not shown), and urinary bladder (**Figure 4q**), which were normalized in i.v.-AAV8hAAT-treated mice. LAMP1 staining of kidneys was also greatly reduced in i.v.-AAV8hAAT treated animals, displaying a complete correction in distal and proximal tubules and a reduction to near normal levels in the renal glomeruli (**Figure 4m–o** and insets).

High sustained circulating sulfamidase activity reduces CNS pathology

Sulfamidase activity was not detected in the brain of MPSIIIA animals treated with either i.v.-AAV8CAG or in the i.m. treatment cohorts (data not shown). Low-but-detectable activity of the enzyme was noted through the entire brain of MPSIIIA male mice treated with i.v.-AAV8hAAT reaching ~10% of the activity of healthy WT mice (**Figure 5a**). This was parallel to a significant decrease of the GAG content through the whole brain (**Figure 5b**).

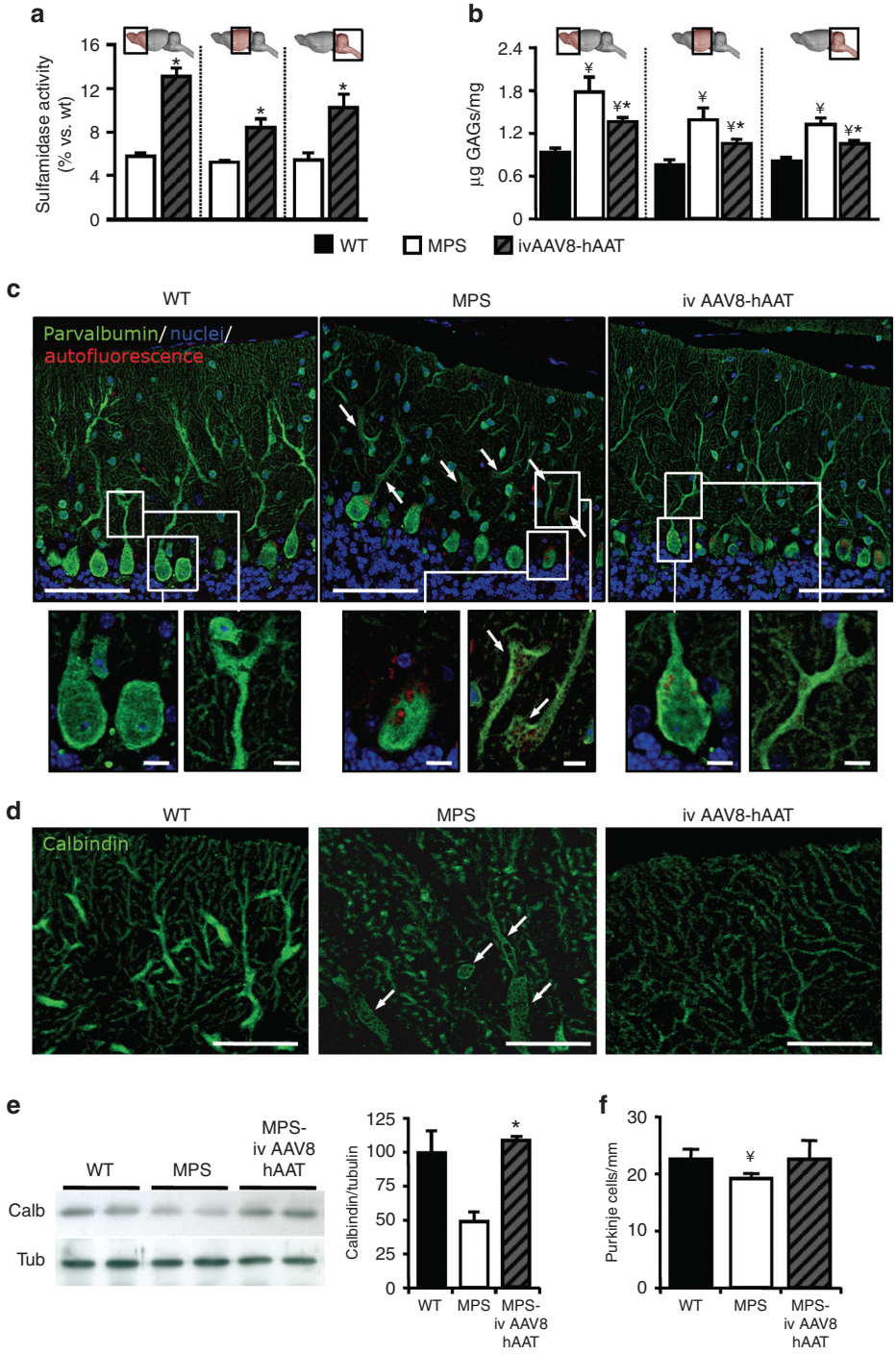

Figure 5 Intravenous AAV8-hAAT treatment ameliorates neurological pathology. (**a**) Percentage of WT sulfamidase activity achieved in the brain of MPSIII mice after i.v.-AAV8hAAT treatment (n = 4–8). The brain regions analyzed are illustrated in the diagrams above the plot. (**b**) GAG storage in brain of i.v.-AAV8hAAT treated male mice. GAG content was normalized to tissue weight. Results are means ± SEM of 4–8 mice per group. (**c**) Parvalbumin immunostaining, which stains Purkinje, basket, and stellate neurons of the cerebellar cortex. Parvalbumin is shown in green, nuclei in blue, and autofluorescence in red. Insets show the accumulation of autofluorescent material in the somas (left insets) and dendrites (right insets) of Purkinje cells in MPSIIIA mice, in which dendrites appeared dilated and vacuolated (white arrows). In treated mice, Purkinje cells accumulated less autofluorescent material, particularly in dendrites, and had an aspect resembling that of WT animals. Bar: 100 µm (insets, 10 µm). (**d**) Calbindin immunostaining of sections of the cerebellar cortex. Calbindin (green), which specifically stains Purkinje cells, allowed a better visualization of the positive effects of the i.v.-AAV8hAAT treatment on Purkinje dendrites (right panel). Bar: 100 µm. (**e**) Western blot analysis of calbindin in cerebellar protein extracts. Representative immunoblot (left) and its densitometric analysis (right) are shown. (**f**) Purkinje cell count. Values are means ± SEM of 3–4 mice per group. $^{¥}P < 0.05$ versus control, $^{*}P < 0.05$ versus untreated MPS. GAG, glycosaminoglycan; hAAT, human α-antitrypsin; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

In contrast, treated females showed little or no differences in brain sulfamidase activity and GAG content compared to nontreated females (**Supplementary Figure S7**). In all experimental cohorts, no sulfamidase mRNA or vector genomes were detected in the brain (data not shown).

Parvalbumin immunohistochemistry stains Purkinje, basket, and stellate neurons (**Figure 5c**). In MPSIIIA mice, cells of the Purkinje layer showed accumulation of autofluorescent material in the somas (middle panel, left insets) and dendrites (middle panel, right insets), which appeared dilated and vacuolated (white arrows). In treated mice, Purkinje cells had an aspect that resembled more that of WT animals and showed less accumulation of autofluorescent material, particularly in dendrites (right panel and insets). High magnification confocal microscopy of calbindin-stained sections, which specifically labels Purkinje cells, demonstrated the same pattern of altered dendrites in untreated MPSIIIA mice, with enlarged and vacuolated dendrites, and the partial reversal of this phenotype in i.v.-AAV8hAAT-treated mice (**Figure 5d**). Western blot analysis demonstrated that the cerebellum of untreated MPSIIIA males had ~50% of the calbindin content of WT, and these levels were completely normalized in treated animals (**Figure 5e**). This twofold decrease in the amount of calbindin could not be explained by a reduced number of Purkinje cells, since although MPSIIIA mice had less Purkinje cells, this reduction was barely of 15%, when compared with WT (**Figure 5f**). No Purkinje cell loss was observed in i.v.-AAV8hAAT treated males.

Electron microscopy analysis of cerebellar cortex from nontreated MPSIIIA mice showed that Purkinje neurons of untreated MPSIIIA mice were filled with many electron-dense inclusions, which varied from small membranous whorls to large granular bodies (**Figure 6a**), as previously described.[4,16] In contrast, Purkinje cells of i.v.-AAV8hAAT-treated mice contained smaller inclusions and in reduced numbers (**Figure 6a**). Enlargement of the smooth endoplasmic reticulum compartment was commonly found both in Purkinje axons and dendrites of nontreated MPSIIIA mice, even forming dense stacks of dilated smooth endoplasmic reticulum (**Figure 6b**). Smooth endoplasmic reticulum cisterns in the axons and dentrites of Purkinje cells had a normal appearance in brain sections of i.v.-AAV8hAAT treated mice, and no abnormal structure was found (**Figure 6b**).

When the histology of the occipital cortex of MPSIIIA mice was analyzed, a strong reactivity for glial fibrillary acidic protein (GFAP), indicative of astrogliosis, and GSI-B4, indicative of activated microglia, was observed (**Supplementary Figure S8**). In i.v.-AAV8hAAT-treated MPSIIIA mice, GFAP and GSI-B4 reactivity was slightly reduced, suggesting partial reversion of the phenotype. When the ultrastructure of the cortex was analyzed [Q7]

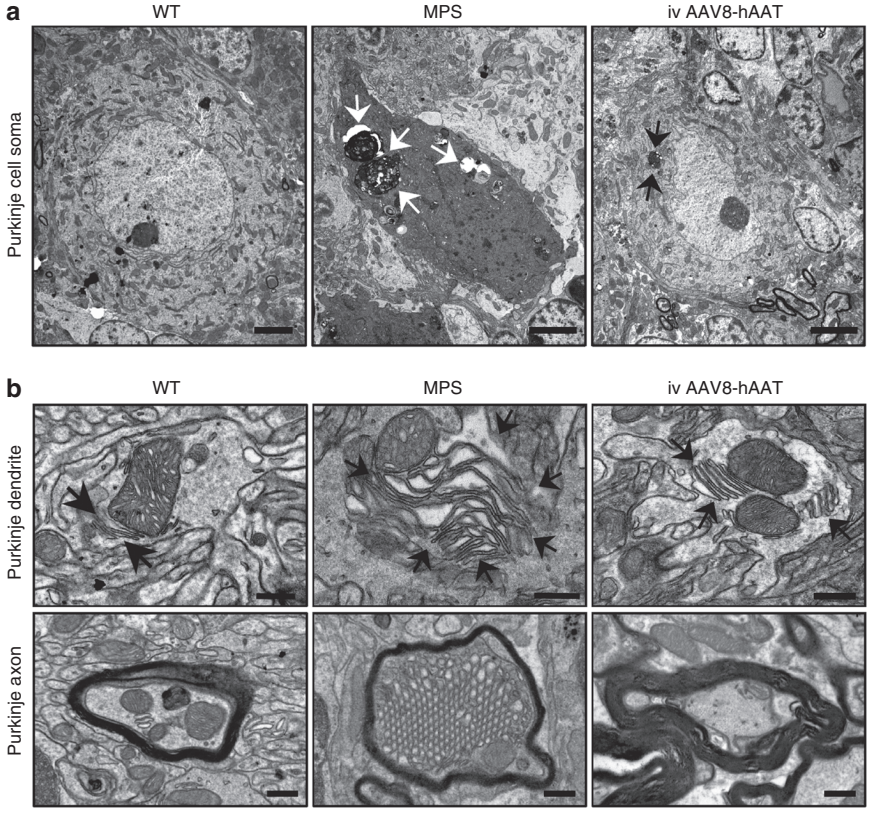

Figure 6 Transmission electron microscopy analysis of the cerebellum. Transmission electron microscopy of the cerebellar cortex from healthy WT, untreated and i.v.-AAV8-hAAT treated MPSIIIA male mice. (**a**) Somas of Purkinje neurons of nontreated MPSIIIA mice were filled with many large electron-dense inclusions (white arrows), whereas in i.v.-AAV8-hAAT treated males less and smaller inclusions were found (black arrows). (**b**) Enlargement of the smooth endoplasmic reticulum found in the axons and dendrites of Purkinje neurons in nontreated mice. Arrows indicate position of the smooth endoplasmic reticulum in dendrites. Bars: 4 µm in **a**, 0.5 µm in **b**. hAAT, human α-antitrypsin; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

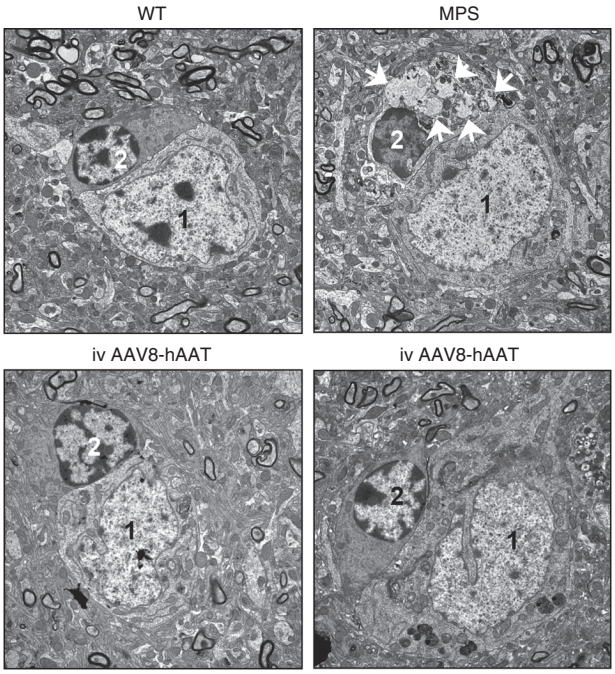

Figure 7 Reduction of lysosomal pathology in glial cells of the occipital cortex. Transmission electron microscopy depicting cortical neurons of the occipital cortex and their associated glial cells. MPSIIIA lysosomal pathology was much more evident in perineuronal glial cells than neurons. Note the presence of large electro-lucent vacuoles in the glial cells from MPSIIIA untreated male samples (white arrows, upper right panel) and not in WT samples (upper left panel). This enlargement of the lysosomal compartment was greatly reduced in i.v.-AAVhAAT-treated mice, and most of the perineuronal glial cells in these samples presented an aspect similar to that of WTs (bottom panels). (i) neuron, (ii) perineuronal glial cell. hAAT, human α-antitrypsin; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

by transmission electron microscopy, we could not detect clear differences in the ultrastructure of occipital cortical neurons among MPS untreated and treated groups, but a clear enlargement of the lysosomal compartment was observed in perineuronal glial cells in MPSIIIA untreated mice (**Figure 7**, top right panel), which was practically absent in samples from i.v.-AAV8hAAT treated animals (**Figure 7**, bottom panels). Altogether, these results suggest that sustained high circulating sulfamidase activity partially prevent neuronal degeneration in MPSIIIA mice.

High sustained circulating sulfamidase activity leads to prolonged lifespan

To assess if the amelioration in CNS histopathology observed in 10-month-old mice translated any improvements in motor coordination, we tested treated mice on the accelerating rotarod. A statistically nonsignificant prolongation of the "time to fall" was observed in i.v.-AAV8hAAT-treated MPS males in comparison with untreated counterparts (P = 0.071, **Figure 8a**). Importantly, treatment with i.v.-AAV8hAAT extended the lifespan of MPSIIIA males (**Figure 8b**). By 17 months of age, all untreated MPS males had died while 100% of i.v.-AAV8hAAT-treated males were still alive (median survival = 14.2 ± 0.5 versus 18.8 ± 0.9 months for MPS untreated and treated males respectively, P = 0.001) (**Figure 8b**). This improvement was not

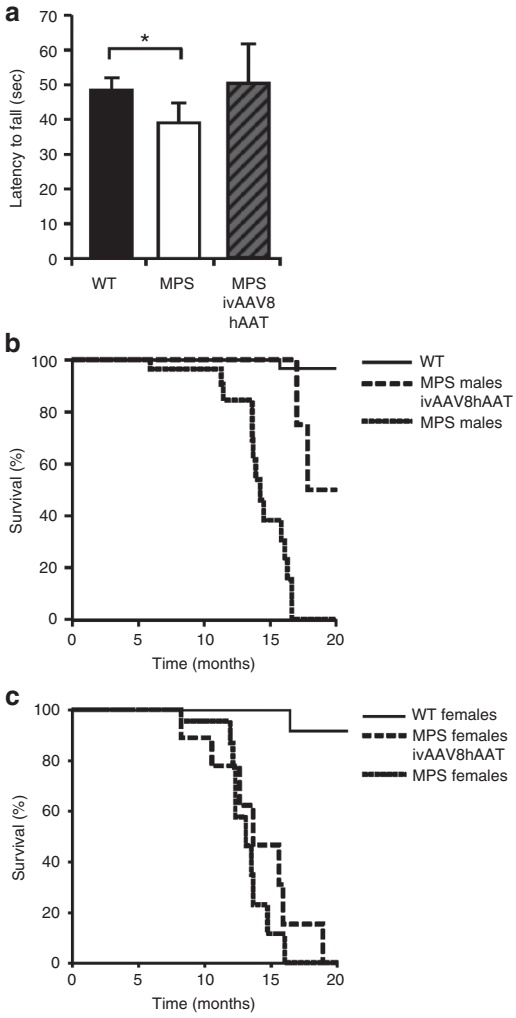

Figure 8 Prolonged survival in i.v.-AAV8hAAT treated males. (**a**) Assessment of locomotor coordination through the rotarod test in 10-month-old males. MPSIIIA mice had a shorter latency to fall than age-matched WT males. Results are mean ± SEM (WT, n = 24; MPS untreated n = 16), P < 0.036. Mice treated with i.v.-AAV8hAAT showed a statistically not significant improvement in the performance of the test with respect to MPSIIIA untreated animals (n = 5, P = 0.071). (**b**) Kaplan–Meier survival analysis in WT (n = 79), MPSIIIA (n = 32) and i.v.-AAV8hAAT-treated males (n = 14). Treatment with AAV-mediated liver-directed gene therapy considerably extended the lifespan of MPSIIIA animals (P = 0.001 for untreated versus treated MPSIII males). (**c**) Kaplan–Meier survival analysis in WT (n = 77), MPSIIIA (n = 33) and i.v.-AAV8hAAT-treated females (n = 13). Treatment with AAV-mediated liver-directed gene therapy did not extend the lifespan of MPSIIIA females (P = 0.467 for untreated versus treated MPSIII female mice). AAV, adeno-associated virus; hAAT, human α-antitrypsin; MPSIIIA, mucopolysaccharidosis type IIIA; WT, wild-type.

evident in the female group (median survival = 13.1 ± 0.5 versus 13.9 ± 1.2 months for MPS untreated and treated females respectively, P = 0.467, **Figure 8c**), a result in agreement with the lower levels of sulfamidase activity measured in serum and brain and the lower degree of GAG reduction observed in female animals (**Supplementary Figure S7a,b**). Therefore, the greater survival of i.v.-AAV8hAAT-treated MPSIIIA males further demonstrated the therapeutic potential of sustained supraphysiological levels of circulating sulfamidase obtained through liver-directed gene transfer.

DISCUSSION

Genetic modification of a target tissue to produce sustained, high levels of circulating sulfamidase may be a useful approach for correcting both somatic and neurological pathological alterations characteristic of MPSIIIA. AAV1- or AAV8-mediated transduction of skeletal muscle was not efficient in secreting sulfamidase into the bloodstream, despite the high levels of sulfamidase activity found in muscle homogenates. This treatment only mediated a partial reduction of GAG accumulation in peripheral organs and resulted in no correction in the CNS. SUMF1 is essential for activating sulfatases, and it has been reported that its expression in muscle is low.[19] The fact that we detected activity in the muscles of AAV1-CAG treated MPSIIIA mice several fold higher than WT levels confirms the capacity of the muscle to generate large amounts of active sulfamidase. Yet, the activity of sulfamidase in the serum of treated animals was barely detectable suggesting that the active sulfamidase produced in the muscle did not reach the bloodstream efficiently. The large size of the dimeric sulfamidase (115 kDa) may explain its inefficient secretion, due to the basal lamina surrounding muscle fibers, in which sulfamidase could remain trapped. Gene transfer to skeletal muscle to secrete proteins into the bloodstream has proven successful for the secretion of relatively small proteins, but not those of high molecular weight.[20–24]

The presence of low-but-detectable levels of sulfamidase expression in liver after intramuscular gene transfer suggests that some of the vector reached the systemic circulation and transduced hepatocytes. Vector leakage after intramuscular administration of AAV vectors has previously been observed in mice and large animals,[23,25] the liver being the primary recipient of the AAV vectors, especially for serotypes, like AAV8, with natural hepatic tropism.

To achieve more efficient secretion of the therapeutic transgene, we focused on the genetic modification of the liver, which is highly proficient in protein secretion[26] and it can be efficiently transduced with AAV vectors.[27] Intravenous administration of sulfamidase-expressing AAV8 vectors into MPSIIIA mice resulted in high levels of sulfamidase activity in liver and serum for at least 8 months. The levels obtained in i.v.-AAV8CAG-treated mice resulted in complete clearance of GAG accumulation in all tissues, with the exception of kidney, which is known to be refractory to correction in several LSD models.[28,29] Higher levels of sulfamidase, obtained with an AAV8 vector expressing sulfamidase under the control of a strong liver promoter, completely corrected the disease in all peripheral tissues, including the kidney, without any signs of toxicity. Previous *in vitro* tissue culture experiments have shown that about 1–5% of normal intracellular enzyme activity is sufficient to correct metabolic effects of lysosomal accumulation in cells from LSD patients.[30] However, in an *in vivo* setting, achieving these intracellular activity levels in all tissues can prove difficult since each tissue may have different blood supply, or display different amounts of M6PR on the cell surface. The pattern of expression of the two existing M6P receptors (CD-MPR and CI-MPR) has already been investigated,[31] but we could not detect any clear correlation between the amount of M6P receptor described for a given tissue by Wenk *et al.* and its degree of lysosomal correction in our studies. Then, other factors may account for the different levels of circulating sulfamidase required to correct each tissue. Although there are no studies evaluating the degree of somatic correction achieved with intravenous ERT in any MPSIIIA animal model, ERT studies for other LSDs suggest that amelioration of the disease phenotype can be easily achieved in most peripheral tissues, although high doses are required for complete correction.[28,29,32] It has been shown that intravenous administration of a lentiviral vector expressing sulfamidase to MPSIIIA mice resulted in correction of the disease phenotype in some somatic tissues, in the absence of detectable levels of sulfamidase in circulation,[17] suggesting that steady low levels of enzyme may result in some therapeutic benefit.

It has been reported that AAV-driven hepatic expression promotes transgene-specific immune tolerance.[33] Tolerance induction may be particularly useful for the treatment of those Sanfilippo A subjects whose causative mutations in the sulfamidase gene are associated with a significant loss of coding information,[34] with consequent lack of tolerance to the therapeutic transgene product. In our results, the sustained expression of sulfamidase for at least 8 months after vector administration suggests the absence of immune responses directed against sulfamidase. However it should also be noted that MPSIIIA mice do have circulating, but inactive, sulfamidase antigen, likely contributing to prevent these responses.[3] In our study, we also observed a remarkable difference in AAV liver transduction between genders, although significant correction of the disease phenotype was achieved even in female mice, which displayed three times less circulating sulfamidase activity than males. The same androgen-driven discrepancy in liver transduction efficiency has been reported for AAV vectors in mice and dogs.[35,36]

Remarkably, here we demonstrate that important amelioration of the CNS pathology is possible with a peripheral gene transfer approach. To date, the only approaches tested to treat neuropathology in MPSIIIA models circumvented the BBB either through the infusion of the recombinant enzyme to the cerebrospinal fluid by intracisternal delivery,[12,37] or through the intracranial administration of AAV vectors.[16] One important complication of CNS-directed ERT strategies is the need of repetitive enzyme administrations into the cisterna magna, which increase the risk of infections and other complications.[38] Intracranial administration of AAV vectors effectively results in the prevention of the neuropathology when applied to newborn MPSIIIA[16] or MPSIIIB mice;[39] however, this approach has not been tested in adult MPSIIIA mice, which already present neurological alterations. In addition, the low diffusion rate of vectors from the injection sites could be a major limitation to scaling up this approach to a human brain, since multiple injections would be required.[40] Here we obtained a homogenous distribution of sulfamidase throughout the entire brain of male MPSIIIA mice through a noninvasive approach. Adult brain BBB is believed to be completely impermeable to lysosomal enzymes, since no amelioration of the neurological symptoms have been observed with ERT in many different LSDs.[10] More recent studies demonstrated that the BBB obstacle could be overcome if large doses of the recombinant enzyme were administered.[11] High protein doses probably saturate the M6PR in the liver, leading to a longer half-life of the circulating protein, which can diffuse into the brain parenchyma through so-called

extracellular pathways.[11] It is known that substances that are not actively transported through the BBB are still present in the cerebrospinal fluid, although at concentration ~200-fold lower than in the serum.[41] Our study suggests that sustained high levels of circulating sulfamidase may lead to a slow but continuous leakage of the enzyme to the brain parenchyma through this nonsaturable mechanism. To enhance the correction of the MPSIIIA neurological phenotype after a liver-directed gene therapy strategy such as the one presented herein, it would be necessary to enhance the access of the circulating active sulfamidase to the CNS. Temporary opening of the BBB can be achieved through chemical methods, such as the intravenous administration of hyperosmotic mannitol. This is a common method in medical practice for CNS delivery of chemotherapeutic drugs;[42] granted that the periodic use of this procedure is compatible with the underlying disease state of MPSIIIA partients, it could be applied to mediate short-term increases of sulfamidase in the CNS. It has also been shown that epinephrine temporarily upregulates M6PR receptor on the BBB, which in turn enables an increased transport of lysosomal enzymes into the brain parenchyma.[43] Another possibility is that the BBB is not completely impermeable in MPSIIIA mice, although previous studies of ERT suggest that the BBB in adult MPSIIIA mice is uncompromised.[44,45] It should be noted that these studies were performed using relatively low doses of the recombinant enzyme, which differ from our results obtained with sustained very high levels of circulating sulfamidase. In agreement with the requirement of continuous supraphysiological levels of circulating sulfamidase to partially revert the MPSIIIA neurological phenotype, we recorded a significant increase in the lifespan of i.v.-AAV8hAAT-treated males. We did not observe a statistically significant amelioration in locomotor coordination in these animals; the use of larger cohorts of animals or the assessment of locomotor coordination at later time points may, however, uncover a beneficial effect of the therapy.

Other mechanisms may also be involved in the amelioration of brain alterations. For example, in MPSIIIA and other LSD it has been suggested that affected neurons constantly release part of their lysosomal content to the extracellular medium[46] and/or to juxtaposed macrophages,[47] which would then drain to perivascular spaces and to systemic circulation. This CNS-derived excess of heparan sulfate could be taken up and degraded by peripheral tissues, which contain high levels of sulfamidase activity, hence creating a "peripheral sink"[48] that may contribute to the observed reduction of brain GAG content.

In summary, we have developed a new noninvasive gene transfer approach for the treatment of MPSIIIA based on the genetic modification of the liver via the intravenous delivery of sulfamidase-expressing AAV8 vectors. Sustained expression of the sulfamidase transgene in liver at high levels effectively reversed GAG storage in somatic tissues, reduced their accumulation in the cerebral cortex and cerebellum ameliorating CNS pathology, and ultimately led to significantly prolonged survival of MPSIIIA male mice.

MATERIALS AND METHODS

Animals. Congenic C57Bl/6 sulfamidase-deficient mice (MPSIIIA) were used.[4] Affected MPSIIIA and healthy control mice were bred from heterozygous founders. Genotype was determined by a PCR on genomic DNA which amplifies a sequence encompassing the mutation, and subsequent digestion with the MspA1I restriction enzyme, as previously described.[3] Mice were fed *ad libitum* with a standard diet (Harlan-Teklad, Indianapolis, IN) and maintained under a light-dark cycle of 12 h. All experimental procedures were approved by the ethics committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

Recombinant AAV vectors. To generate AAV vectors carrying sulfamidase gene, the complementary DNA of murine sulfamidase was cloned into an AAV backbone plasmid under the control of the ubiquitous hybrid promoter CAG (containing the chicken β-actin promoter and cytomegalovirus enhancer) or the liver-specific hAAT promoter.[18] Vectors were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications.[49] Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Lowell, MA) in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and then cotransfected with: (i) a plasmid carrying the expression cassette flanked by the viral inverted terminal repeats (ITRs) (described above); (ii) a helper plasmid carrying the AAV rep2 and the correspondent cap (cap1 and cap8) genes; and (iii) a plasmid carrying the adenovirus helper functions. Plasmids carrying the adenovirus helper functions and cap genes as well as hAAT promoter were kindly provided by K.A. High, Children's Hospital of Philadelphia. Vectors were purified by two consecutives cesium chloride gradients using either a standard protocol or an optimized protocol as previously described.[49] Vectors were dialyzed against phosphate-buffered saline, filtered, titrated by quantitative PCR and stored at −80 °C until use.

Vector administration and sample collection. For intramuscular injections, MPSIIIA animals of 2–3 months of age were anesthetized with a mixture of ketamine (100 mg/kg) and xylacine (10 mg/kg), and a total dose of 10^{12} vector genomes of the appropriate AAV vector were injected into six muscles (30 µl/muscle) of the hindlimbs (quadriceps, gastrocnemius, and tibialis anterior from both legs). For intravenous delivery of AAV vectors, a total dose of 10^{12} vector genomes of the appropriate AAV vector were injected into 2–3-month-old MPSIIIA animals via tail vein. At 10 months of age, mice were anesthetized and then transcardially perfused with 10 ml of phosphate-buffered saline to completely clear blood from tissues. The entire brain and multiple somatic tissues (including liver, spleen, pancreas, kidney, lung, heart, skeletal muscle, and testicles) were collected and either frozen in liquid nitrogen and stored at −80 °C or immersed in formalin for subsequent histological analyses.

RNA analysis. Total RNA was obtained from skeletal muscle and liver with TriPure Isolation Reagent (Roche, Mannheim, Germany) and analyzed by Northern blot. Blots were hybridized with a murine sulfamidase probe, labeled with ^{32}P-dCTP by random priming with Ready-to-Go DNA Labelling Beads (Amersham Biosciences, Buckinghamshire, England), and exposed for a short period of time that allowed the detection of exogenous but not endogenous mRNA.

Sulfamidase activity and GAG quantification. Liver, skeletal muscle, and brain samples were sonicated in water and sulfamidase activity was assayed in supernatants with the fluorogenic substrate 4-methylumbelliferyl-α-D-N-sulphoglucosaminide (or MU-GlcNS) (Moscerdam Substrates, Oegstgeest, Netherlands) as described previously.[50] Sulfamidase activity levels were normalized against the total amount of protein quantified using the Bradford protein assay (Bio-Rad, Hercules, CA). For GAG quantification, tissue samples were weighted and then digested with proteinase K and extracts were clarified by centrifugation and filtration. GAG levels in tissue extracts and urine were determined using Blyscan sulfated glycosaminoglycan kit (Biocolor, Carrickfergus, UK) with chondroitin 4-sulfate as the standard. GAG levels in tissues were normalized to wet tissue weight and in urine to creatinine concentration, measured with a colorimetric kit (ABX Diagnostics, Montpellier, France).

Histological analyses. Tissues were fixed for 12–24 h in 4% formaldehyde, embedded in paraffin and sectioned. For immunohistochemical detection of LAMP1, Mac2, parvalbumin and calbindin, paraffin sections were incubated overnight at 4 °C with rat anti-LAMP1 antibody (1D4B; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Mac2 (ab53082; Abcam, Cambridge, MA), rabbit anti-calbindin D28k (Swant, Bellinzona, Switzerland), rabbit anti-parvalbumin (Swant), rabbit anti-GFAP (Z0334; Dako, Glostrup, Denmark) or with Lectin GSI-B4 (L5391; Sigma, St Louis, MO). Secondary antibodies were: biotinylated rabbit anti-rat antibody (Dako) or biotinylated goat anti-rabbit IgG (Vector laboratories, Burlingame, CA). Brightfield sections were stained with 3,3-diaminobenzidine (Sigma), counterstained with hematoxylin, and images were obtained with an Eclipse E800 optical microscope (Nikon, Tokyo, Japan). For fluorescence sections, streptavidin-conjugated Alexa 488 (Molecular Probes, Eugene, OR) or streptavidin-Alexa 568 (Molecular Probes) were used for visualization. Nuclei were stained with TOPRO-3, and images were obtained with a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Measurement of the hepatocyte area. Hematoxylin/eosin staining of liver sections was performed. The mean area of 200 hepatocytes from four different images per animal was determined using a Nikon Eclipse E800 microscope (Nikon) connected to a videocamera and an image analyzer (analySIS 3.0; Soft Imaging System, Lakewood, CO).

Purkinje cell count. Hematoxylin/eosin staining of two brain sections per animal was performed. The length of the Purkinje cell layer and the number of Purkinje neurons of each section was determined using a Nikon Eclipse E800 microscope (Nikon) connected to a videocamera with a color monitor and an image analyzer (analySIS 3.0). Purkinje cell count was defined as the number of Purkinje cells per millimetre of Purkinje cell layer.

Western blot analysis. Halves of cerebellum were homogenized in protein lysis buffer. Ten micrograms of protein were run on a 10% (wt/vol) SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes and probed overnight at 4 °C with primary antibodies against calbindin (Swant) and α-tubulin (Abcam). Detection was performed using horseradish peroxidase-labelled swine anti-rabbit antibody (Dako) and ECL Plus Western blotting detection reagent (Amersham Biosciences).

Transmission electron microscopic analysis. Mice were killed by an overdose of isofluorane (Isofluo; Esteve, Barcelona, Spain) and perfused via inferior vena cava with 2.5% glutaraldehyde and 2% paraformaldehyde. A small portion (~1 mm^3) of the lateral lobe of the liver and of the culmen of the cerebellum were sectioned and incubated for 2 hours at 4 °C in the same fixative. Specimens were postfixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, and then dehydrated and embedded in epoxy resin. Ultrathin sections (600–800 Å) were stained using lead citrate and examined with a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Biodistribution. Total DNA was isolated with MasterPureDNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Vector genome copy number in 20 ng of genomic DNA was determined by quantitative real-time PCR with a set of primers specific for the hAAT promoter. Forward primer: 5′-GAT CTT GCT ACC AGT GGA ACA G-3′; reverse primer: 5′-TGA GTC AGA CAG TCT CTG GGA G-3′ (Invitrogen, Carlsbad, CA). Serial dilutions of a linearized plasmid bearing the hAAT-sulfamidase expression cassette used in the study supplemented with 20 ng of irrelevant mouse genomic DNA were used to build a reference standard.

Locomotor function. Animals were tested on an accelerating rotarod (Rotarod LE8200; Panlab, Barcelona, Spain). Before the trial, mice were gently placed on the rod with the speed set at 4 rpm and trained to remain on the rod for 2 minutes. After training, mice were given three consecutive trials in which the rod accelerated from 4–40 rpm in a 5-minute interval. Latencies to fall from the rod were recorded and the mean of all three trials was used for analysis.

Statistical analysis. All results are expressed as mean ± SEM. Statistical comparisons were made using either *t*-test or one-way analysis of variance. Statistical significance was considered if $P < 0.05$. Survival analysis was done by the Kaplan–Meier method and the log-rank test was used for comparisons.

SUPPLEMENTARY MATERIAL

Figure S1. Somatic and neurological pathway in 2-month-old MPSIIIA.
Figure S2. Expression of sulfamidase in the skeletal muscle leads to high intramuscular sulfamidase activity but low secretion to serum.
Figure S3. Reversal of lysosomal accumulation in the liver.
Figure S4. Correction of hepatomegaly.
Figure S5. Correction of GAG storage in testicle.
Figure S6. LAMP1/Mac2 immunostaining.
Figure S7. Sulfamidase activity and CAG storage in CNS of female mice.
Figure S8. Astrogliosis and microgliosis in the cerebral cortex of MPSIIIA mice.
Table S1. AAV8 vector distribution in somatic tissues.

ACKNOWLEDGMENTS

We thank Federico Mingozzi for helpful scientific discussions, and Xavier Leon, Marta Moya, Verónica Melgarejo, and Ángel Vázquez for technical assistance. A.R., X.M.A., and A.R. were recipients of predoctoral fellowships from Ministerio de Ciencia e Innovación (Spain) and P.V. from Fundación Ramón Areces. This work was supported by grants from MPS España and Instituto de Salud Carlos III (PI 061417), Spain and from European Community (CLINIGENE LSHB-CT-2006-018933). The authors declared no conflict of interest.

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