A single injection of double-stranded adeno-associated viral vector expressing GH normalizes growth in GH-deficient mice

Alessia Sagazio*, Xiao Xiao¹, Zhong Wang^{1,†}, Marco Martari and Roberto Salvatori

Division of Endocrinology, Department of Medicine, Johns Hopkins University School of Medicine, 1830 East Monument Street #333, Baltimore, Maryland 21287, USA

¹Division of Molecular Pharmaceutics, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599, USA

(Correspondence should be addressed to R Salvatori; Email: salvatori@jhmi.edu)

*(A Sagazio is on leave from Department of Endocrinology and Metabolism, University G D'Annunzio, Chieti 66100, Italy)

†(Present address of Z Wang is at Department of Surgery, The Brooklyn Hospital Center, 121 Dekalb Avenue, Brooklyn, New York 11201, USA)

Abstract

GH is secreted by the somatotrophic cells of the pituitary gland, and its deficiency (GHD) impairs longitudinal growth. Due to its short half-life, GH therapy needs administration of GH injections daily. Adeno-associated viral vectors (AAV) can deliver gene therapy to animals with possible future applications in humans. The new generation of double-stranded AAV vectors (dsAAV) provides widespread, strong, and stable transgene expression without toxicity and immune response. To determine whether such a new system could be used to deliver GH to a mouse model of isolated GHD due to ablation of the GHRH knock-out gene (GHRHKO), we have created AAV viral particles containing mouse GH cDNA driven by a cytomegalovirus promoter (dsAAV8-CMV-GH), and tested them in male GHRHKO mice. GHRHKO animals received either a single (low dose) or

two (high dose) i.p. injections of dsAAV8-CMV-GH (1×10^{11} particles) at the 10th and 11th days of age, or a placebo injection, and were followed up to the 6th or 24th week of life. A single dsAAV8-GH injection caused body length and weight normalization. At week 6, serum GH was higher in mice receiving both virus doses compared with controls, while it was normal at week 24. Serum IGF-1 increased in both virus-treated groups, and it was normal at 24 weeks. GH mRNA expression was detected in liver, skeletal, and heart muscle of virus-injected animals. These data show that normalization of longitudinal growth can be reached in GHD mice using a single injection of a double-stranded adeno-associated virus expressing GH.

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Introduction

Growth hormone (GH) is secreted into the systemic circulation by the somatotrophic cells of the anterior pituitary gland under the stimulatory control of the hypothalamic factor GH-releasing hormone (GHRH). GH acts both directly and indirectly through the mediation of insulin-like factor-I (IGF-I; Le Roith *et al.* 2001, Flint *et al.* 2003, Salvatori 2004). GH-deficient (GHD) children have growth impairment and metabolic alterations, which are reversed by replacement treatment with GH (rtGH; Bryant *et al.* 2002, Hindmarsh & Dattani 2006). However, due to the very short half-life of GH (Giustina & Veldhuis 1998), rtGH requires daily s.c. injections (Saggese *et al.* 1998). Different rodent models of isolated GHD (IGHD) have been used to study the effects of rtGH on a variety of biological parameters (Beamer & Eicher 1976, Groesbeck *et al.* 1987, Woodall *et al.* 1991, Coschigano *et al.* 2003). We have recently developed a mouse model of IGHID by generalized ablation (knock-out, KO) of the GHRH gene (GHRHKO; Alba & Salvatori 2004). We have shown that in GHRHKO animal somatic growth and body composition abnormalities can be normalized by daily injections of recombinant mouse GH (Alba *et al.* 2005a).

Viral vectors provide more long-term effect than traditional drug therapy and represent an alternative efficient mechanism of delivery of proteins. Gene therapy approach to GHD animals has been studied using recombinant adenovirus vectors encoding murine or rat GH and single-stranded adeno-associated viral vectors (ssAAV; Hahn *et al.* 1996, Marmay *et al.* 1999, Rivera *et al.* 1999). The recombinant adenovirus used in the past often elicited a potent immune response, sometimes requiring administration of anti-inflammatory drugs to virus-treated animals (Adesanya *et al.* 1996), although with more modern vectors this problem is reduced (Alba *et al.* 2005b). In addition, the conventional AAV vectors tend to have delayed and less effective gene transfer and expression (Wang *et al.* 2003) because AAV DNA is packaged and delivered as a single-stranded genome, which is transcriptionally inactive until converted into double-stranded (ds) template. Recent research has focused on preparation of AAV vectors that can package a self-complementary dsDNA obtaining rapid, strong, and stable expression in animals (dsAAV; Berns & Hauswirth 1979, Berns & Giraud 1995, Samulski *et al.* 1999). This overcomes the rate-limiting step of conversion of single into dsDNA. Non-pathogenicity, non-toxicity and lack of immune response, robust infectivity,

and long-term gene transfer identify the new AAV vectors approach. AAV vectors are derived from the replication-defective parvovirus (Berns & Hauswirth 1979, Berns & Giraud 1995, Samulski *et al.* 1999). The vectors carrying a foreign gene can infect both dividing and non-dividing cells *in vitro* and *in vivo*, obtaining a long-term transgenic expression minimizing the toxicity and cellular immune response (Ferrari *et al.* 1996, Afione *et al.* 1999, Bangari & Mittal 2006), resulting in robust and long-term delivery to many tissues, including muscle (Wang *et al.* 2005), liver (Song *et al.* 2001), and pancreas (McClane *et al.* 1997, Ayuso *et al.* 2004). These dsAAV vectors have successfully delivered gene therapy in animal models of diabetes (McClane *et al.* 1997, Ayuso *et al.* 2004, Rehman *et al.* 2005, Wang *et al.* 2006), muscular dystrophy (Zhu *et al.* 2005), hemophilia (Arruda *et al.* 2005), loss of corneal endothelium (Lai *et al.* 2005), and cerebral ischemia (Tsai *et al.* 2002), with possible important future applications in humans.

We have investigated whether such a new delivery system could be a tool to administer GH to GHD animals. We show that normalization of longitudinal growth can be achieved in GHD mice using a single injection of a dsAAV expressing mouse GH cDNA.

Materials and Methods

Generation of the dsAAV8-cytomegalovirus-GH (AAV8-CMV-GH) vector

The AAV8-CMV-green fluorescent protein (GFP) vector (dsAAV8-CMV-GFP) was described earlier (Xiao *et al.* 1998, Gao *et al.* 2002). Full-length mouse cDNA was amplified by PCR from pituitary cDNA of a C57BL6 mouse using primers 5'-TTGGGGTCGAGGAAAACAGGTA-3' and 5'-GATG-CATCTTAATTTTATTAGAGC-3', and inserted in pCR2.1 TA plasmid (Invitrogen). The dsAAV8-CMV-GH plasmid was generated by replacing the GFP gene of the dsAAV8-CMV-GFP (Wang *et al.* 2005) using BamHI/NotI restriction sites. The recombinant viral stocks were produced by the adenovirus-free, triple plasmid cotransfection method (Xiao *et al.* 1998). The AAV particles were subsequently purified by two rounds of CsCl gradient ultracentrifugation. The titer of viral genome

particle number was determined by quantitative DNA dot bolt method (Snyder *et al.* 1996).

Animals and vector administration

We used homozygous GHRHKO male mice and heterozygous male carriers as normal controls to guarantee similar genetic background (mixed C57BL6/129SV; Alba & Salvatori 2004). Following guidance from previous studies (McClane *et al.* 1997, Song *et al.* 2001, Tsai *et al.* 2002, Ayuso *et al.* 2004, Arruda *et al.* 2005, Lai *et al.* 2005, Rehman *et al.* 2005, Zhu *et al.* 2005, Wang *et al.* 2006), dsAAV8-CMV-GH viruses were injected intraperitoneally at the 10th day of age. Treatment groups are described in Table 1. Low dose (LD) groups (12 GHRHKO pups per group) received 1×10^{11} particles of dsAAV8-CMV-GH viral particles; High dose (HD) group (6 GHRHKO pups) received 1×10^{11} particles of dsAAV8-CMV-GH at the 10th and 11th days of life (high dose); GFP group (6 GHRHKO pups) received 1×10^{11} particles of dsAAV8-CMV-GFP; and placebo groups (Plac) (12 GHRHKO pups per group) received placebo injections. Control groups (Ctl) consisted of 12 pups heterozygous (HTZ) for GHRHKO gene. HD, GFP, and one half (six mice) of LD, Plac, and Ctl groups were killed at the 6th week of age. The other half of LD, Plac, and Ctl groups were allowed to reach the 24th week of age (long-term groups).

All pups were weaned at the 4th week of age and housed based on treatment. All mice experienced a controlled environment with 14 h light:10 h darkness cycles at 21 °C and 23% humidity, food standard mouse/rat (ProLab RMH2500; PMI Nutrition International, Brentwood, MO, USA), and water *ad libitum*. All the procedures were approved by the Johns Hopkins Institutional Animal Care Committee.

Auxological data

Starting from the first week of life, all pups were examined weekly for 5 weeks (LD, HD, GFP, Plac, and Ctl groups) or for 23 weeks (LD, Plac, and Ctl long-term groups) by measuring total body weight (TBW) using a daily-calibrated electronic balance (Scout Pro Balance; Ohaus Corp SP601, Pine Brook, NJ, USA) and body length (nose-to-anus distance, N-A), using an electronic

Table 1 Treatment schedule

Animals		Treatment	Age at killing
Group			
LD	GHRHKO (n=12)	1×10^{11} particles of dsAAV8-CMV-GH	6 pups at 6 weeks 6 pups at 24 weeks (long-term group)
HD	GHRHKO (n=6)	2×10^{11} particles of dsAAV8-CMV-GH	6 weeks
GFP	GHRHKO (n=6)	1×10^{11} particles of dsAAV8-CMV-GFP	6 weeks
Plac	GHRHKO (n=12)	Placebo	6 pups at 6 weeks 6 pups at 24 weeks (long-term group)
Ctl	HTZ (n=12)	Placebo	6 pups at 6 weeks 6 pups at 24 weeks (long-term group)

n, the number of mice in each group; HTZ, heterozygotes for the GHRHKO allele.

Figure 1 Body length was (nose-to-anus distance N-A, mm) assessed weekly for the first 6 weeks (1A) and 24 weeks of life (B). Error bars represent standard deviations. (A) Final length in LD group (treated with low-dose dsAAV8-CMV-GH) was not different from the Ctl group (control). The HD group reached a longer length than the LD and Ctl groups (** $P < 0.05$). The GFP group was statistically different compared with the LD, HD, and Ctl groups (* $P < 0.01$). (B) The LD group reached the same length as the control group (Ctl) and the animals were statistically longer than the placebo-treated GHRHKO mice (Plac) (* $P < 0.05$).

digital caliper (Fisher brand Traceable Digital Caliper; Fisher Scientific, Hampton, NH, USA). Two days before the end of the study, animals were anesthetized using Avertin (tribromoethanol; Sigma-Aldrich) and ~200 μ l blood were collected with heparinized capillaries by retro-orbital bleed for GH and IGF-I measurements. Following the same method at the 10th, 14th, 18th, 22nd weeks of life, we collected blood from four mice of LD and Ctl long-term groups for IGF-I measurements. Serum was stored at -20°C until the day of assay. Mice were killed by halothane overdose. After killing, we harvested heart, liver, spleen, right kidney, right testis, and right gastrocnemius muscle. Organs were weighed using an Ohaus Adventurer Pro Analytical Balance (AV264 Ohaus, Pine Brook, NJ, USA). Femur and tibia length were measured using an electronic caliper after dissection of the surrounding tissues and careful disarticulation of the bones. Right femur length was considered as the maximal distance between the head of the great trochanter and the distal condyles, while right tibia length as the maximal distance between proximal condyles and malleolus. To determine the effects of the GH expression on body composition, animals were skinned, the perirenal and

epididymal fat pads were pooled (visceral fat, VF), while the sum of fat pads from the interscapular and axillary region, thighs, and inguinal region was considered s.c. fat (SF). Lean mass (LM) was measured by weighing animals deprived of tail, skin, adipose tissue, and internal organs. LM, VF, and SF weights of each animal were normalized to TBW by calculating the percentage as follows: (weight (g)/TBW (g)) $\times 100$.

Detection of GH RNA expression

Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's recommendations. We used 25/50 mg of tissue/mice from liver, heart, right gastrocnemius muscle, spleen, right kidney, and right testis. Total RNA was quantified spectrophotometrically at 260 nm (DU 640 Spectrophotometer; Beckman-Coulter, Fullerton, CA, USA). One microgram of total RNA was used to generate cDNA using reverse transcriptase (Moloney murine leukemia virus (M-MLV) Reverse Transcriptase; Promega). Control tubes were used without reverse transcriptase (reverse transcriptase negative).

Figure 2 Total body weight (g) assessed weekly for the first 6 weeks (A) and 24 weeks of life (B). Error bars represent standard deviations. (A) Both groups treated with dsAAV8-CMV-GH normalized in TBW compared with the Ctl group. The GFP group (dsAAV8-CMV-GFP) was significantly different from the LD, HD, and Ctl groups (* $P < 0.01$). (B) At the 24th week of age the weight of the LD group normalized compared with HTZ mice (Ctl) and was higher than the placebo-treated GHRHKO mice (Plac) (* $P < 0.01$).

GH cDNA was amplified by PCR using primers described above, expected to generate an 859 bp band corresponding to the full-length mouse GH cDNA.

Serum hormones measurements

We measured serum GH and IGF-I at the 6th week of age in LD, HD, GFP, Plac, and Ctl groups. In addition, IGF-I serum levels were measured at the 10th, 14th, 18th, and 22nd weeks of age in four mice from LD and Ctl, and at the 24th week of age in long-term LD, Plac, and Ctl groups. GH serum levels were measured at the 24th week in four mice from LD, Plac, and Ctl groups.

Serum GH was measured by RIA (Rat GH RIA, RGH-45HK; Linco-Millipore, Billerica, MA, USA). The standard curve of the assay performed in accordance with the manufacturer's provided samples. Each sample was assayed in duplicate.

Serum IGF-I was measured using mouse/rat IGF-I kit (DSL-2900; DSL Webster, TX, USA), after acid-ethanol extraction, following the manufacturer's recommendations. The assay included quality controls provided by the manufacturer. The standard curve of the assay performed in accordance with the manufacturer's provided samples. Each sample was assayed in duplicate.

Fluorescence analysis

Tissues were fixed in 4% paraformaldehyde (PFA, pH 7.3), washed in 0.2 M PO_4 buffer, and conserved -20°C in Tissue-Tek OCT Compound. Transversal sections (7 μm) were cut from frozen tissue. All tissues were placed on glass slides and covered by 25 μl Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA) containing propidium iodine for direct evaluation of GFP expression under the fluorescence microscope.

Statistical analysis

Data were analyzed by ANOVA using the SPSS statistical package (SPSS Inc., Chicago, IL, USA), with *post hoc* analysis using Bonferroni's method. Data were considered statistically significant at $P < 0.05$.

Results

Auxological data and body composition

As shown in Fig. 1A and B, all groups of GHRHKO mice injected with dsAAV8-CMV-GH virus reached significant

Figure 3 Femur and tibia length (mm) at the time of killing: 6 weeks (LD, HD, GFP, Plac, and Ctl groups) (A and B) and 24 weeks (LD, Plac, and Ctl groups) (C). Error bars represent standard deviations. (A and B) At the 6th week of age, there was no difference between both dsAAV8-CMV-GH-treated groups (LD and HD) compared with the control group (Ctl). Both groups were longer compared with the placebo group (Plac) (* $P < 0.05$). (C) Similar results were observed at the 24th week: no difference existed between the dsAAV8-CMV-GH-treated group (LD) and the HTZ control group (Ctl). * $P < 0.05$ versus placebo (Plac).

increase in the final N-A length (6 and 24 weeks). At the 6th week of age N-A length of HD group was significantly higher than LD and Ctl groups. Weight normalized in all virus-treated groups at the 6th and 24th weeks of age (Fig. 2A and B). As shown in Fig. 3A–C, both low- and high-dose-treated groups at the 6th and 24th weeks of age normalized in femoral and tibial lengths.

At the end of both periods of observation (6 and 24 weeks) body composition (Fig. 4) and weights of all organs (Table 2) were normalized in both dsAAV8-CMV-GH-treated groups, with the exception of the spleen that was heavier in the high-dose than in the Ctl group. Spleen was also heavier in treated mice than HTZ controls at 24th week. Kidneys were significantly heavier in the HD group compared with LD group.

GH mRNA expression

GH mRNA was detected by RT-PCR only in liver, skeletal, and heart muscle of virus-injected animals both at the 6th (Fig. 5A) and 24th weeks (Fig. 5B), but not in spleen, kidney, and testis.

Serum GH measurement

As shown in Fig. 6A, at 6 weeks of age GH levels of low- and high-dose groups were significantly higher than levels of HTZ

control mice. Serum GH levels of control mice were higher than placebo- and GFP-treated animals. At 24 weeks of age, serum GH levels of virus-treated mice were higher than placebo but not different from HTZ mice (Ctl; Fig. 6B).

Serum IGF-I measurement

As shown in Fig. 7A, at 6 weeks of age, serum IGF-I levels were significantly higher in the high-dose-treated group compared with heterozygous mice, but no statistically significant difference was observed between low dose and HTZ controls. Serum IGF-I levels were significantly higher in both groups treated with dsAAV8-GH virus than in placebo-treated GHRHKO mice and mice treated dsAAV8-CMV-GFP. There was no statistical difference between serum IGF-I levels in the low-dose-treated group and HTZ mice at the 10th, 14th, 18th, 22nd, and 24th weeks of age, and they were both higher than in placebo-treated GHRHKO mice at week 24 (Fig. 7B).

Fluorescence analysis

Microscopy analysis showed that in GHRHKO mice injected with dsAAV8-CMV-GFP, GFP was expressed in liver, skeletal, and (to a lesser extent) heart muscle, while no detectable

Figure 4 Body composition at the 6th week (upper panel) and 24th week (lower panel). Error bars represent standard deviations. Absolute and relative s.c. fat (SF), visceral fat (VF), and lean mass (LM) is shown. At both time points, virus-treated GHRHKO mice (LD and HD groups) showed normalization in absolute and relative SF, VF, and LM compared with the HTZ group (Ctl). * $P < 0.02$ versus placebo groups (Plac).

expression was observed in spleen and kidney in agreement with the result of GH mRNA in mice injected with dsAAV8-CMV-GH (Fig. 8A). No fluorescence was observed in tissues from HTZ mice (Fig. 8B).

Discussion

The aim of this study was to test whether GH expression driven by new generation of dsAAV8 vector could be an effective delivery system to administer GH to GHD animals. The choice of the dsAAV8 vectors, the dosage of the virus particles, and the i.p. route of administration were based on previous studies performed with the same viral delivery method and same promoter (McClane *et al.* 1997, Song *et al.* 2001, Tsai *et al.* 2002, Ayuso *et al.* 2004, Arruda *et al.* 2005, Lai *et al.* 2005, Rehman *et al.* 2005, Zhu *et al.* 2005, Wang *et al.* 2006). The choice of HTZ animals as control is based on their normal growth pattern (Alba & Salvatori 2004), and the need to use animals with genetic background similar to the GHD mice. Our data confirm long-term expression of the dsAAV8 transgene for up to 22 weeks after a single

treatment. At the 6th week of age (1 month and 2 days after the injection of the virus particles), GH-deficient mice treated with low- and high-dose dsAAV8-CMV-GH (LD and HD groups) had significantly higher GH serum levels than control mice, achieving significant growth increase when compared with placebo-treated animals. Indeed, the high-dose group reached N-A length that was even longer than HTZ controls. Both low- and high-dose treatments followed to the 6th week of life, and low dose followed to the 24th week of age, achieved complete normalization of femoral and tibial lengths, confirming proper biological functioning of the product of the expressed GH. Weight measurement of individual organs (liver, kidney, spleen, heart, and testis) paralleled the effects of GH on longitudinal growth. Body composition also showed normalization of percentage fat mass induced by GH, as shown previously in the same animals after daily treatment with exogenous recombinant mouse GH (Alba *et al.* 2005a). We observed a disproportionate increase in spleen weight induced by high dose of the virus. A previous study has shown that rats treated with recombinant GH display increased spleen mass (Vickers *et al.* 2002). Since GH plays an important role in controlling hematology

Table 2 Organ weights (mg) at the time of killing

Group	Liver (mean $\pm$ S.D.)	Spleen (mean $\pm$ S.D.)	Heart (mean $\pm$ S.D.)	Kidney (mean $\pm$ S.D.)
LD ($n=6$)	1034.4 $\pm$ 78.4 [†]	69.7 $\pm$ 18.8 [†]	145.7 $\pm$ 29.6 [†]	136.3 $\pm$ 16.2 [†]
HD ($n=6$)	1238.5 $\pm$ 28.1 [†]	127.0 $\pm$ 17.3 ^{†,§}	163.58 $\pm$ 10.7 [†]	189.00 $\pm$ 9.3 ^{†,‡}
GFP ($n=6$)	484.9 $\pm$ 23.5*	32.1 $\pm$ 4.6*	92.9 $\pm$ 4.6*	92.4 $\pm$ 6.3*
Plac ($n=6$)	462.1 $\pm$ 38.6	45.2 $\pm$ 7.6	74.2 $\pm$ 11.7	78.80 $\pm$ 13.5
Ctl ($n=6$)	1142.4 $\pm$ 53.2	68.1 $\pm$ 6.5	146.7 $\pm$ 14.0	159.2 $\pm$ 10.8
LD ($n=6$; long-term group)	1398.6 $\pm$ 56.9	128.7 $\pm$ 23.2 ^{,¶}	226.8 $\pm$ 26.8	225.7 $\pm$ 32.7
Plac ($n=6$; long-term group)	529.8 $\pm$ 25.1	33.3 $\pm$ 5.2	96.3 $\pm$ 16.5	86.4 $\pm$ 10.5
Ctl ($n=6$; long-term group)	1360.7 $\pm$ 23.3	75.3 $\pm$ 14.9	207.1 $\pm$ 18.3	233.2 $\pm$ 7.6

n , the number of the mice in each group. *Denotes $P<0.1$ versus LD, HD, and Ctl groups. [†]Denotes $P<0.1$ versus GFP and Plac groups. [‡]Denotes $P<0.1$ versus LD group. [§]Denotes $P<0.1$ versus LD, HD, GFP, Plac, and Ctl groups. ^{||}Denotes $P<0.1$ versus Plac long-term group. [¶]Denotes $P<0.1$ versus Ctl long-term group.

(Hanley *et al.* 2005), we hypothesize that high GH serum levels caused proliferation of splenic tissue.

The detection of normal levels of serum GH and IGF-I at 24 weeks of life (22.5 weeks after the injection of the virus) confirmed previous studies showing that this viral vector has stable and long-term expression limited to the liver, skeletal, and cardiac muscle (McClane *et al.* 1997, Song *et al.* 2001, Tsai *et al.* 2002, Ayuso *et al.* 2004, Arruda *et al.* 2005, Lai *et al.* 2005, Rehman *et al.* 2005, Zhu *et al.* 2005, Wang *et al.* 2006). This is confirmed by both detection of GH mRNA and fluorescence studies in mice injected with GFP-expressing vector. The pattern of expression (as shown by GH mRNA) was similar at 6 and 24 weeks, suggesting stable and long-term gene expression after the initial infection. Our results are similar to previous works that has used single-stranded adenoviral vector that caused expression of recombinant GH up to 7 weeks (Hahn *et al.* 1996). In that paper, however, expression beyond 7 weeks was not assessed, and tissue expression of the virus was not examined. Another GH delivery system using a single-stranded adeno-associated virus has been shown to last up to 10 months after a single injection,

but it is used in nude mice unable to mount an immune response (Rivera *et al.* 1999). Therefore, our results show that in GHD immunocompetent mice a species-specific GH cDNA continue to exert biological activity up to 6 months from a single injection.

Serum levels of GH at 6 weeks in the low-dose group were higher than at 24 weeks, showing that the degree of GH expression reduces over time. This reduction did not reflect a parallel decrease in serum IGF-I. This is not completely surprising. Serum IGF-I reflects mostly liver IGF-I production, and in the past we have noted a frequent discrepancy between the effects of GH or GHRH on growth and serum IGF-I, which seems to be a less than optimal index of GH

Figure 5 GH mRNA was detected by RT-PCR only from the liver (L), skeletal (M), and heart (H) muscle of the LD group at the 6th week of age (5A) and LD group at the 24th week (5B), but not in the spleen (S), kidney (K), and testis (T). Pituitary RNA of normal mouse was used as a positive control (C). PhiX-174 RF DNA-Hae III digest was used as DNA marker.

Figure 6 Serum GH at the 6th (A) and 24th (B) weeks of age. Error bars represent standard deviations. (A) Serum GH levels in the LD and HD groups were higher compared with all other groups ($*P<0.05$). (B) At the 24th week serum GH was normalized in dsAAV8-CMV-GH-treated group compared with the Ctl group, but still higher than GHRHKO placebo (Plac) ($*P<0.05$ versus Plac).

Figure 7 Serum IGF-I at the 6th week of age (A) and at the 10th, 14th, 18th, 22nd, and 24th weeks of age (B). Error bars represent standard deviations. (A) IGF-I was higher in the HD group (high dose) compared with the Ctl group (* $P<0.05$). The LD group is significantly different in IGF-I levels compared with GHRHKO mice (Plac) (** $P<0.05$). (B) The LD group was not different compared with the HTZ mice (Ctl) but was different at the 24th week of age compared with the GHRHKO mice (Plac) (* $P<0.05$).

effect in mice (Alba *et al.* 2005a, Fintini *et al.* 2005a,b). Indeed, in mice with liver-specific ablation of the IGF-I gene, somatic growth is essentially normal despite markedly reduced serum IGF-I (Yakar *et al.* 1999). Nevertheless, the fact that serum

IGF-I levels at 24 weeks are significantly higher in virus-treated than in placebo-treated GHRHKO mice shows that the expressed GH is still functional, and that no resistance to its effect developed over time. This is not surprising, as we

Figure 8 Fluorescence microscopy. (A) At the 6th week dsAAV8-CMV-GFP-injected GHRHKO mice showed fluorescence in liver (A), gastrocnemius (B), and (to a lesser extent) heart (C) muscle. Blue coloring is DAPI nuclear staining. There is no expression of GFP viral vector in the spleen (D) and kidney (E). (B) No fluorescence was observed in tissues from HTZ mice (A1, liver; B1, gastrocnemius; C1, heart; D1, spleen; E1, kidney). All photographs of the tissues were taken with 2 s of exposure time under fluorescence microscope.

have used a species-specific GH cDNA, and previous reports describing the development of anti-GH antibodies in GH-treated GHD rodents used exogenous GH from different species (Groesbeck & Parlow 1987). As we do not have a group that was allowed to survive longer than 6 months, we cannot determine whether eventually the transgene expression would disappear. However, long-term gene expression over a year mediated by AAV vectors in experimental animals has been well documented in different animal species using various transgenes (Rivera *et al.* 2005). The positive effect of the dsAAV virus on growth was not accompanied by any readily evident side effect, despite the fact that GH expression was not physiologically regulated. Obviously, the use of a universal promoter is a limitation to any possible clinical application of this approach, due to the well-known long-term risks associated with excessive and unregulated GH secretion as seen in patients with GH-secreting pituitary adenomas (Melmed 2006). In addition, cardiac expression and possible long-term effects of locally produced GH on the heart muscle is also a concern (Lombardi *et al.* 1997). Therefore, future studies are being designed using either inducible promoters (that may be regulated at will), similarly to the data published by Rivera *et al.* (1999), or promoters that are specific for a given tissue (e.g. muscle) which may allow significant systemic delivery despite limited local expression. Finally, the occurrence of circulating anti-AAV antibodies has been reported in rodents (Peden *et al.* 2004) and liver enzyme increase has been reported in human hemophilic subjects treated with intrahepatic AAV vector expressing factor IX (Manno *et al.* 2006). All these factors may limit future clinical use of this approach.

While the applicability of the findings of this work is very far from any conceivable clinical application in humans, the advantages of new dsAAV vectors offer a good starting point for the development of novel regulated viral gene delivery systems for GH administration.

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