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Partial correction of the dwarf phenotype by non-viral transfer of the growth hormone gene in mice: Treatment age is critical

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ABSTRACT

Non-viral transfer of the growth hormone gene to different muscles of immunodeficient dwarf (lit/scid) mice is under study with the objective of improving phenotypic correction via this particular gene therapy approach. Plasmid DNA was administered into the exposed quadriceps or non-exposed tibialis cranialis muscle of lit/scid mice followed by electroporation, monitoring several growth parameters. In a 6-month bioassay, 50 μg DNA were injected three times into the quadriceps muscle of 80-day old mice. A 50% weight increase, with a catch-up growth of 21%, together with a 16% increase for nose-to-tail and tail lengths (catch-up = 19–21%) and a 24–28% increase for femur length (catch-up = 53–60%), were obtained. mIGF1 serum levels were ~7-fold higher than the basal levels for untreated mice, but still ~2-fold lower than in non-dwarf scid mice. Since treatment age was found to be particularly important in a second bioassay utilizing 40-day old mice, these pubertal mice were compared in a third bioassay with adult (80-day old) mice, all treated twice with 50 μg DNA injected into each tibialis cranialis muscle, via a less invasive approach. mIGF1 concentrations at the same level as co-aged scid mice were obtained 15 days after administration in pubertal mice. Catch-up growth, based on femur length (77%), nose-to-tail (36%) and tail length (39%) increases was 40 to 95% higher than those obtained upon treating adult mice. These data pave the way for the development of more effective pre-clinical assays in pubertal dwarf mice for the treatment of GH deficiency via plasmid-DNA muscular administration.

1. Introduction

Plasmid-based gene therapy is among the most popular non-viral systems used in clinical trials, being particularly effective when DNA injection is followed by electroporation. Its utilization has continued to increase, representing 18.3% of all gene therapy clinical trials in 2012, close behind adenovirus (23.3%) and retrovirus (19.7%) applications [1,2]. Concerning systemic protein delivery, quite successful clinical improvements have been reported for the treatment of peripheral artery diseases and critical limb ischemia [3–5], but the utilization of naked DNA is, as far as we know, still at the preclinical level for the treatment of systemic diseases [6]. The first clinical tests for electroporation in humans, mostly related to DNA vaccination, were carried out by injection into the deltoid muscle [7–9] or into the anterior thigh [10], a muscular compartment that includes the quadriceps. This type of treatment is in general safe, well tolerated and clinically acceptable, even if a small percentage of the subjects may experience some adverse reaction, pain or discomfort [7].

In order to develop appropriate preclinical testing protocols for non-viral gene therapy in skeletal muscle, in our recent studies we have utilized the immunodeficient (lit/scid) and the immunocompetent (lit/lit) dwarf mice as suitable models of systemic diseases like growth hormone deficiency (GHD), since these mutants have an inherited growth defect strictly resembling human isolated type 1B GHD [11–13]. Indeed, our research group has found significant body weight increases of approximately 23% and 34% in 1 month- and 3 month-bioassays in which hGH- or mGH-plasmid DNA were injected into the exposed quadriceps muscle of lit/scid and lit/lit mice, respectively [14–16]. Because the saline-treated lit/scid mice presented a more stable body weight and a higher catch-up growth was obtained upon GH-DNA treatment (27% in the 1 month-treatment) in comparison with lit/lit mice (16% in the 3 month-treatment), the former model was chosen for the present work, the goal of which was to maximize the phenotypic correction of dwarfism via a non-viral GH gene transfer. This was tried first by increasing treatment time (up to 6 months), with repeated injections, always monitoring hGH and mIGF1 levels, and then

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by testing different age groups. A comparative study of different growth parameters was also carried out, especially considering those directly related to linear growth.

2. Materials and methods

2.1. Animals

The mutant strains of CB17-Ghrhr lit/+ Prkdc scid/Bm (lit/scid) and scid mice were obtained from Dr. W. Beamer (The Jackson Laboratory, Bar Harbor, ME, USA) [17]. The animals were maintained on a vented shelf and used to breed colonies of lit/scid and scid mice, as previously described [14]. Animals between 40 and 80 days of age were used for the experiments, which were approved by the local animal ethics committee.

2.2. Plasmid

The plasmid pUC-UBI-hGH was derived from a standard pUC-19 cloning plasmid by including the ubiquitin C promotor (position -1464 to - 15) upstream to a 2152 bp BamHI - EcoRI fragment of the hGH gene containing 4 introns and polyadenylation sequences [18]. The ubiquitous expression of various genes driven by this promoter has been reported by different authors [19,20] and its efficiency in hGH skeletal muscle expression already demonstrated by us in previous work [14]. The plasmid was multiplied in DH5alfa bacteria and purified using the DNA Xtra Midi/Maxi-Nucleobond Machnerey-Nagel purification system (Duren, Germany).

2.3. Plasmid administration and electroporation

The animals were anesthetized with xylazine and ketamine, followed by a hyaluronidase (20 U/20 μL) injection into the exposed quadriceps muscle region, as described [14]. After 30 min, 50 μg/20 μL of purified plasmid was administered in the same region, followed by electrotransfer with eight 90 V/cm pulses of 20 ms, separated by 0.5 s intervals, using an ECM-830 electroporator and a caliper electrode with a 3 mm distance between the plates (length/size 1.0 × 1.0 cm), both from BTX (Holliston, MA, USA). Saline was used as the control in all assays. For non-exposed tibialis cranialis electroporation, mice were treated with hyaluronidase as the above followed by one 800 V/cm pulse of 100 μs and by one 100 V/cm pulse of 400 ms.

2.4. Bioassay procedures

Three different Bioassays were carried out. In Bioassay I (6-month assay) all animals were ~80 days old at the onset of the experiment. One group of lit/scid (n = 26) received doses of 50 μg (1st and 2nd administration) or 100 μg/mouse (3rd administration) of pUC-UBI-hGH, while a second group (n = 24) received saline. Both groups were submitted to electroporation into the right (day 1) or into the left (day 104 and 161) exposed quadriceps. A third group (n = 21), composed of co-aged scid mice, was used as the positive control and for catch-up growth calculation.

In Bioassay II (1-month assay) one group of lit/scid, 40-day old at the onset of the experiment, was treated only once with 50 μg of pUC-UBI-hGH via quadriceps administration. A second group, also 40-day old, received only saline and was also submitted to electroporation. All groups consisted of 7 mice.

In Bioassay III (2-month assay) one group of lit/scid was 40-day old and the other 80-day old at the onset of the experiment. Both groups received twice (on day 1 and on day 41) 50 μg/20 μL of pUC-UBI-hGH, injected into each tibialis cranialis muscle. A third and a fourth group, with either 40- or 80-day old mice, received only saline and were also electroporated. A fifth and a sixth group consisting of co-aged scid mice was used as the positive control and for catch-up growth calculation. The number of mice per group is reported in Table 3.

The body weight of the animals was determined throughout the entire assay period and used to calculate the average daily weight variation. The animals whose blood was withdrawn from the retro-orbital cavity during the experiment were sacrificed in order to continue the experiment with only completely healthy mice. The tail and nose-to-tail lengths were measured with an electronic caliper before and at the end of the experiment. Blood was collected, femurs were dissected and measured with the same caliper. Serum mIGF1 levels were measured using the Quantikine mouse-rat IGF1 kit (R&D Systems, MN, USA), while serum hGH concentrations were determined utilizing an in-house radioimmunoassay, based on NIDDK reagents (Dr A.F. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA) [21].

2.5. Catch-up growth calculation

Catch-up growth (C-uG) was calculated, as previously reported [16], using the body weight (g), nose-to-tail (cm), tail (cm), femur (mm) lengths or mIGF1 concentration, according to the following formula:

$$\text{C-uG} = (\text{Wt} - \text{Wc})/(\text{Wn} - \text{Wc}) \times 100.$$

where:

Wt = final weight or length or mIGF1 concentration of the treated group;

Wc = final weight or length or mIGF1 concentration of the control (saline − treated) group;

Wn = final weight or length or mIGF1 concentration of a normal co − aged animal group (scid mice in this case).

2.6. Statistical analyses

Quantitative variables, reported as the mean ± SD, were analyzed by the unpaired Student's t test. Growth equations were generated by fitting the data to a quadratic relationship. The quadratic, linear and independent coefficients, calculated from the different experimental groups, were compared via the F-test method included in the Prism 5.0 package (GraphPad Software Inc., La Jolla, CA, USA). Means and curves were considered to be statistically different, representing distinct treatment effects, when the P value was <0.05.

3. Results

The results of the 6-month assay (Bioassay I) in which three progressive administrations of hGH-DNA were applied to the right and left quadriceps of lit/scid mice DNA are shown in Fig. 1. The weight increase reached about 50% on the initial value, body and tail length presented the same increment of 16%, and the right and left femurs increased 28% and 24%, respectively (Table 1). Fig. 2 illustrates the different dissected femurs where the length increase due to hGH-DNA treatment in lit/scid mice, as well as the natural growth occurring in non-dwarf scid mice, can be visually appreciated. Based on all the parameters presented in Table 1, the catch-up growth over the 6-month bioassay was between 19 and 60%, particularly high especially considering femur measurements. An integrated statistical analysis based on the catch-up growth parameter is also presented: it shows a highly significant difference in the increase between weight, nose-to-tail and tail versus right and left femur measurements. As shown in Fig. 1, treated lit/scid and co-aged scid mice underwent similar growth in terms of absolute body mass variation. Considering that the non-dwarf mice were already ca. 3-fold heavier than dwarf mice at the beginning of the experiment and that both groups consisted of adult animals, it was then decided to experiment the DNA treatment on younger animals. In Table 2 the corresponding mIGF1 determinations point to an increase in the

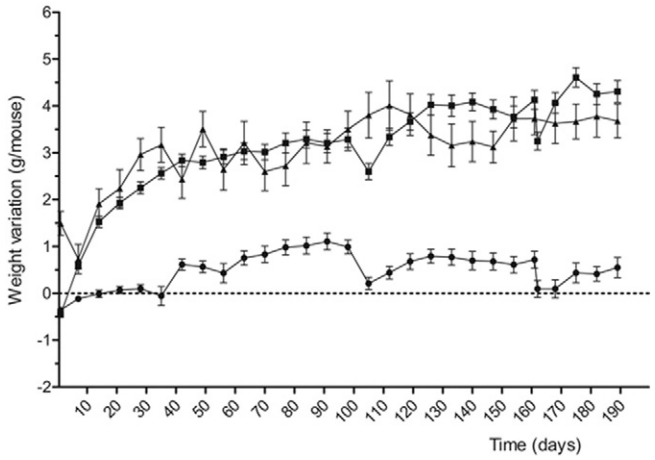

Fig. 1. Bioassay I. Weight variation in a 6-month bioassay with 80-day old lit/scid mice treated twice with 50 μg and once with 100 μg of hGH plasmid DNA (-■-) and of saline-treated lit/scid (-●-) and untreated co-aged scid mice (-▲-). Equations for each curve, adjusted to the quadratic relationship $Y = B_0 + B_1X + B_2X^2$: a) saline: $Y = -0.2238 + 0.01882X - 8.75.10^{-5}X^2$ (DF = 27; $R^2 = 0.6230$) b) hGH DNA final: $Y = 0.6050 + 0.04323X - 13.54.10^{-5}X^2$ (DF = 27; $R^2 = 0.8625$) 1st month: $Y = -0.3004 + 0.1537X - 221.8.10^{-5}X^2$ (DF = 3; $R^2 = 0.9661$) c) untreated scid: $Y = 1.219 + 0.03556X - 12.74.10^{-5}X^2$ (DF = 26; $R^2 = 0.7424$) Obs. The difference between curves a and b is statistically significant ($P < 0.0001$).

concentration of this analyte in treated animals towards the end of the experiment, reaching a value of 242 ng/mL. This value is about 7-fold higher than that in untreated co-aged lit/scid, but still 1.8-fold lower than that in co-aged non-dwarf scid mice. hGH was also measured, providing circulating levels of approximately 1.5 ng/mL from the 1st to the 5th month, but reaching 3 ng/mL at the end of the treatment (6th month).

To test the influence of mice younger age, a new 30-day assay (Bioassay II) was thus carried out in 40-day old mice using one single hGH-DNA administration under the usual treatment conditions. The corresponding growth curve is presented in Fig. 3 showing that this younger group is experiencing a quite dramatic weight increase, with a growth curve slope (B_1) of 0.2969 g/mouse/day. In comparison, after the 1st month, 80-day old mice experienced, in Bioassay I, a weight increase of 0.1537 g/mouse/day. The mIGF1 levels were 17.7 ± 13.8 and 125.6 ± 48.0 ng/mL for saline-treated and 40-day old hGH-treated lit/scid mice, respectively (n = 7 in both cases).

Taking advantage of a recently developed methodology (Cecchi, manuscript in preparation) based on hGH-DNA injection and electroporation of non-exposed tibialis cranialis muscles, a final experiment (Bioassay III) was carried out in order to directly compare adult and pubertal mice. Fig. 4 presents the results of a 60-day bioassay with two groups of animals that started the treatment at either 40 or 80 days of age and that received two administrations of hGH-DNA. As shown in Table 3, the linear growth parameters for nose-to-tail, tail and femur length exhibited increases of 19–24% for the 40 day old mice. These values were 1.2–2.0-fold higher than those of 80 day old mice. Remarkably higher (1.4- to 1.9-fold) catch-up growth of 36–76% was also obtained in pubertal animals, with the highest phenotypical correction being attained by the femur. When comparing these data with those from Bioassay I, we can observe that the growth response obtained in 60 days in younger animal was even higher than that obtained in adult animal in a 3-fold longer period. The integrated statistical analysis based on catch-up growth confirmed a highly significant difference (P = 0.0002) between 40-day and 80-day old treated mice, considering all measured parameters, mIGF1 included. Even without considering mIGF1, the statistical difference was still highly significant: P = 0.0129. As indicated in Table 4, on day 15, dwarf mice initially 40 days old reached the same mIGF1 levels as non-dwarf mice (non-significant difference: P > 0.05). This was not the case for dwarf mice that were initially 80 days old, in which the

Table 1
Growth parameters determined on lit/scid mice during a 6-month assay (Bioassay I) based on three hGH plasmid administrations to the quadriceps muscle.

Growth parameter and treatment group	Before treatment (X ± SD)	After 1 month (X ± SD)	Increase (%)	After 2 months (X ± SD)	Increase (%)	After 3 months (X ± SD)	Increase (%)	After 5 months (X ± SD)	Increase (%)	After 6 months (X ± SD)	Increase (%)	Final Catch-up (%)
Body weight (g)												
Saline	9.06 ± 1.15	9.34 ± 0.32	8.6	8.01 ± 2.85	−11.6	10.00 ± 0.98	10.4	7.54 ± 0.88	5.3	10.05 ± 0.65	10.9	21.3 ± 4.68
hGH-DNA	9.32 ± 0.99	11.33 ± 2.56	21.6	11.67 ± 1.10	25.2	11.67 ± 0.82	25.2	12.64 ± 0.99	35.6	13.95 ± 0.86	49.7	
Untreated Scid	25.31 ± 3.08	27.16 ± 5.05	7.3	27.60 ± 2.79	9.0	26.89 ± 3.86	6.2	29.79 ± 1.74	17.7	28.32 ± 2.93	11.9	
Nose-to-tail (cm)												
Saline	12.41 ± 0.60	12.55 ± 0.57	1.1	11.37 ± 1.01	−8.4	12.27 ± 0.38	−1.1	12.48 ± 0.23	0.7	12.81 ± 0.38	3.2	21.0 ± 4.71
hGH-DNA	12.14 ± 0.41	12.50 ± 0.83	3.1	13.47 ± 0.45	11.0	13.17 ± 0.28	8.5	13.71 ± 0.34	12.9	14.13 ± 0.30	16.4	
Untreated Scid	17.31 ± 0.05	18.67 ± 0.68	7.9	18.30 ± 0.17	5.7	17.17 ± 0.31	2.7	18.78 ± 1.02	8.5	19.10 ± 0.62	10.3	
Tail (cm)												
Saline	6.04 ± 0.25	6.06 ± 0.46	0.3	5.53 ± 0.40	−8.4	5.81 ± 0.23	−3.8	6.24 ± 0.24	3.3	6.32 ± 0.16	4.6	18.7 ± 5.71
hGH-DNA	5.98 ± 0.21	6.39 ± 0.71	6.9	6.33 ± 0.15	5.9	6.50 ± 0.24	8.7	6.63 ± 0.48	10.9	6.96 ± 0.20	16.4	
Untreated Scid	8.76 ± 0.13	9.17 ± 0.45	4.7	9.25 ± 0.21	5.6	8.93 ± 0.25	1.9	9.43 ± 0.22	7.6	9.75 ± 0.26	11.3	
Right femur (mm)												
Saline	10.6 ± 1.3	9.7 ± 0.6	−8.5	9.5 ± 1.4	−10.4	10.2 ± 1.0	−3.8	10.4 ± 0.5	−1.9	10.4 ± 5.4	−1.9	60.4 ± 8.46
hGH-DNA	10.6 ± 1.3	10.4 ± 0.6	−1.9	13.3 ± 1.7	25.5	13.3 ± 0.7	25.5	13.5 ± 7.9	27.4	13.6 ± 4.5	28.3	
Untreated Scid	12.8 ± 0.1	14.8 ± 0.8	15.6	16.9 ± 0.4	32.0	16.0 ± 1.0	25.0	16.2 ± 4.5	26.6	15.7 ± 11.7	22.7	
Left femur (mm)												
Saline	10.6 ± 1.3	9.8 ± 0.3	−7.6	10.0 ± 1.0	−5.7	9.9 ± 1.1	−6.6	10.5 ± 2.6	−0.9	10.3 ± 4.8	−2.8	52.7 ± 12.76
hGH-DNA	10.6 ± 1.3	10.0 ± 0.0	−5.7	13.1 ± 0.3	23.6	11.6 ± 0.7	9.4	12.6 ± 11.9	18.9	13.2 ± 7.0	24.5	
Untreated Scid	12.8 ± 0.0	15.7 ± 1.5	22.7	16.8 ± 0.3	31.2	15.0 ± 0.0	17.2	16.1 ± 2.0	25.8	15.8 ± 7.1	23.4	

Integrated statistical analysis based on catch-up growth

Body weight/nose-to-tail length/tail length			Right femur/left femur			P-value
Average ± SD	CV (%)	n	Average ± SD	CV (%)	n	
20.39 ± 5.05	24.8	39	56.71 ± 11.36	20.6	26	<0.0001

Obs. Before treatment: n = 24 lit/scid for saline; n = 26 lit/scid for hGH-DNA; n = 21 scid for hGH-DNA; n = 21 scid mice. During treatment: n = 3 at all times. At the end of the treatment: n = 12 lit/scid for saline; n = 13 lit/scid for hGH-DNA; n = 9 scid mice.

Fig. 2. Example of dissected right femurs of hGH-DNA-treated and saline-treated lit/scid and of untreated scid mice during Bioassay I. ls, untreated lit/scid; sc, non-dwarf scid mice; s, saline-treated lit/scid; p, plasmid DNA-treated lit/scid.

mIGF1 levels were much lower (74 ng/mL), confirming previous data obtained by treating adult lit/scid [15]. On the other hand, there was a significant difference ($P < 0.05$) between the mIGF1 levels of 40- and of 80-day old treated animals, even though with a similar hGH expression. As shown in Table 4, younger heterozygous mice (scid) had lower mIGF1 levels than adults. A highly significant correlation was in fact confirmed between the mIGF1 serum concentration and age (55–120 days) of non-dwarf scid mice, according to the equation: $Y_{ng/ml} = 3.328\ X_{days} + 100.3$ ($n = 21$, $r = 0.776$, $P < 0.001$).

A comparison of the precision/variation of the measurement of each parameter was also performed based on the coefficients of variation (CVs). Four different experiments and 30–44 average values calculated for each one of the 4 parameters (body weight, nose-to tail, tail and femur lengths), measured on the same animal, were considered. The "highest precision/lowest variation" found was for the nose-to-tail measurements, since 82% of the calculated averages provided CVs <5%. Tail length came soon after (70%), then femur (33%) and finally body weight (9%). Moreover, only 45% of CVs related to body weight were <10%, while 100%, 98% and 65% of the CVs were <10% for the nose-to-tail, tail and femur measurements, respectively.

Table 5 presents a comparison between the natural increase of each parameter for untreated lit/scid and scid mice, aged between 2 and 9 months. Lit/scid mice did not show any significant body weight or femur length increase over this period, while their nose-to-tail and tail lengths increased significantly. During the same period, non-dwarf scid mice always showed highly significant growth increases ($P < 0.001$) in all four parameters considered here.

Table 2
Plasma mIGF1 concentrations for lit/scid mice during Bioassay I in comparison with co-aged scid mice.

Treatment group	n	Age (months)	mIGF-I (ng/mL) ± SD	CV	Statistical significance versus saline-treated group
Untreated					
lit/scid	3	3	27.7 ± 9.3	33.6	–
Saline-treated lit/scid					
Month 1	3	4	21.8 ± 7.5	34.5	–
Month 2	3	5	24.7 ± 1.5	6.2	–
Month 3	3	6	27.0 ± 5.0	18.5	–
Month 5	3	8	27.3 ± 9.8	35.9	–
Month 6	8	9	36.1 ± 13.5	37.4	–
hGH-DNA-treated lit/scid					
Month 1	3	4	146.7 ± 73.2	49.9	$P < 0.01$
Month 2	3	5	119.2 ± 26.5	22.2	$P < 0.001$
Month 3	3	6	161.7 ± 18.9	11.7	$P < 0.001$
Month 5	3	8	158.3 ± 54.8	34.6	$P < 0.01$
Month 6	8	9	241.6 ± 67.4	27.9	$P < 0.001$
Untreated scid					
Month 1	3	4	683.3 ± 52.0	7.6	$P < 0.001$
Month 2	3	5	716.7 ± 142	19.8	$P < 0.005$
Month 3	3	6	485.0 ± 78.6	16.2	$P < 0.001$
Month 5	3	8	472.5 ± 74.3	15.7	$P < 0.001$
Month 6	7	9	443.9 ± 97.9	22.1	$P < 0.001$

4. Discussion

A complete correction of the dwarf phenotype has been reported in several mice models by different authors, via adenoviral or adeno-associated viral administration or even by plasmid DNA hydrodynamic transfer [22–25]. A similar correction has never been achieved, however, via intramuscular gene transfer by plasmid DNA administration. This non-viral method would be quite advantageous, considering its ability to transfer large size genes, lower toxicity and cost, ease of preparation, accessibility and possibility of readministration; it also deserves further attention because it can readily satisfy biosafety concerns in clinical trials [2,26–29]. A relevant, though partial, phenotypic correction of dwarfism was attained in the present work via hGH plasmid administration to immunodeficient dwarf mice, with linear catch-up growths of 36–77% depending on the parameter analyzed. These values were reached by treating 40-day old pubertal mice, with the highest correction occurring in the femur length. An integrated statistical analysis based on catch-up growth, calculated for all measurements, was in fact particularly useful to confirm the importance of treatment age. Quite intriguing was the evident superimposition of the two growth curves for treated lit/scid and untreated co-aged scid mice, presented in Fig. 1. Both groups apparently experienced the same absolute mass gain, even though lit/scid mice started the experiment with an average body weight of only ~9 g, while scid mice weighed ~25 g initially.

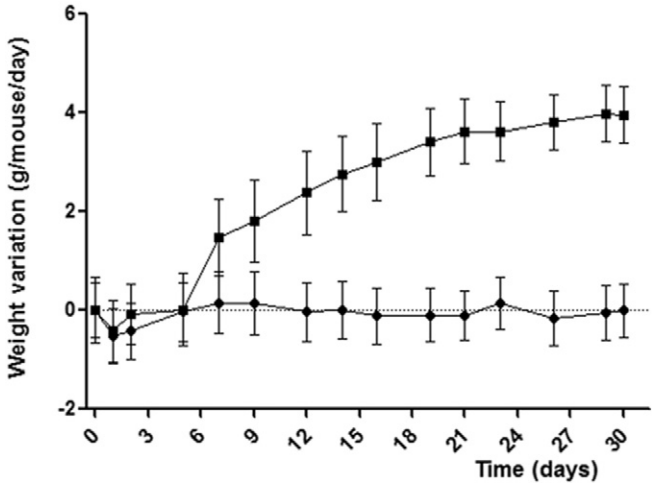

Fig. 3. Bioassay II. Weight variation in a 30-day bioassay with 40 day old lit/scid mice (—■—) treated only once with 50 μg of hGH plasmid DNA via quadriceps injection, $n = 7$ and of saline-treated co-aged lit/scid mice (—●—), $n = 7$. Equations for each curve: a) saline $Y = -0.2457 + 0.02841X - 78.73.10^{-5}X^2$ (DF = 12; $R^2 = 0.2034$) b) hGH-DNA $Y = -0.5571 + 0.2969X - 486.3.10^{-5}X^2$ (DF = 12; $R^2 = 0.9717$) Obs. The difference between curves a and b is statistically significant ($P < 0.0001$).

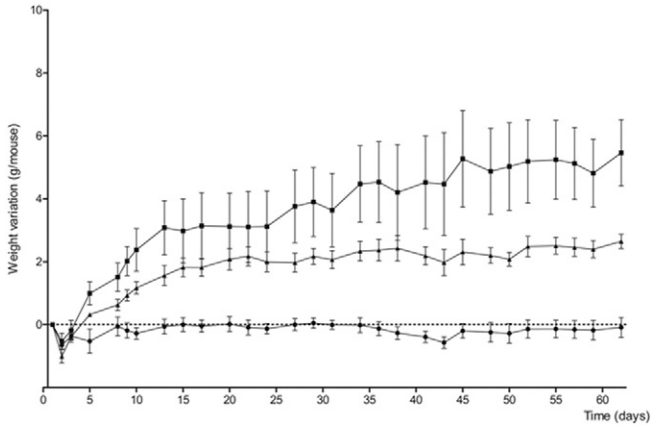

Fig. 4. Bioassay III. Weight variation in a 60-day bioassay with 40 (—■—) or 80 (—▲—) day old lit/scid mice, treated twice with 50 μg of hGH plasmid DNA injected into both tibialis cranialis muscles and of saline-treated lit/scid (—●—). Equations for each curve: a) saline: $Y = -0.2584 + 0.00738X - 12.74.10^{-5}X^2$ (DF = 27; $R^2 = 0.0355$) b) hGH-DNA, 40-day old, final: $Y = 0.2065 + 0.1811X + 168.5.10^{-5}X^2$ (DF = 27; $R^2 = 0.9393$) 1st month: $Y = -0.3577 + 0.3219X - 645.7.10^{-5}X^2$ (DF = 13; $R^2 = 0.9464$) c) hGH-DNA, 80-day old, final: $Y = -0.1182 + 0.1189X + 135.5.10^{-5}X^2$ (DF = 27; $R^2 = 0.8479$) 1st month: $Y = -0.6303 + 0.2330X - 485.8.10^{-5}X^2$ (DF = 13; $R^2 = 0.9392$) Obs. The difference between curves b and c is statistically significant ($P < 0.0001$).

Considering that the treated lit/scid mice were ~80 days old and therefore already postpubertal at day zero, we observed that the treatment in itself could be much more successful if carried out on younger, pubertal animals, as was subsequently confirmed.

Mouse IGF1 proved to be an efficient pharmacodynamic marker for GH action and consequent growth, confirming the data reported by Bielohuby et al. for hypophysectomized and dwarf rodents [30]. It was evident from Bioassay III that the younger initial age is itself responsible for 3.3-fold higher mIGF1 levels, which are direct indicators of the growth effect. Moreover, mIGF1 levels that were not significantly different from those of co-aged non-dwarf mice were achieved by treating pubertal dwarf mice according to the proposed methodology.

Comparing our data with literature reports on non-viral plasmid-DNA transfer of the GH gene in dwarf rodents, Anwer et al. [31] obtained a significant growth increase and IGF1 levels of ~145 ng/mL (control: 35 ng/mL) via tibialis cranialis muscle injection of the plasmid pSK-hGH-GH/PVP-complex in hypophysectomized rats. Complete normalization of growth and of IGF1 levels was described by Sondergaard et al. [24] and Khamaisi et al. [32], who reported IGF1 levels of 400–600 ng/mL (control <100 ng/mL) via hydrodynamic administration of plasmid-DNA in hypophysectomized mice. The intra-muscular administration of plasmid GHRH-DNA carried out by the Draghia-Akli group [33,34], which resulted in important increases of IGF1 levels is also worth mentioning, although these data do not refer to dwarf animals. On the other hand, the viral transfer of the GH gene described by Hahn et al. [22] provided a complete phenotypic correction of dwarfism and normalization of IGF1 serum levels by tail vein adenoviral vector administration in lit/lit mice (238 ng IGF1/mL; control, 61 ng/mL). Almost complete weight normalization and elevated serum mGH were also obtained by Marmary et al. [23] by injecting mGH-encoding adenoviruses into the quadriceps of Snell dwarf mice, although IGF1 levels were determined only in normal young rats submitted in parallel to the same treatment. Finally, Sagatio et al. [25] and Martari et al. [35], from the same research group, used an AAV vector to deliver the mGH gene to a mouse model of Isolated Growth Hormone Deficiency (GHRHKO). The first of these two studies, carried out via intraperitoneal administration, reported full body weight and IGF1 normalization (400–600 ng/mL; control, ~100 ng/mL), while the second, via right quadriceps injection, only provided partial phenotypic correction, with much lower IGF1 levels (~90 ng/mL) that were not significantly different from the control.

Table 3
Parameters directly related to longitudinal growth measured before and after a 60-day assay (Bioassay III) based on tibialis cranialis hGH-DNA administration in 40-day and 80-day old lit/scid mice.

Growth parameter and treatment group	n	Before treatment ($x \pm SD$)	After treatment ($x \pm SD$)	Increase (%)	Catch-up (%)
Nose-to-tail length (cm)					
40-day old, saline	4	10.79 ± 0.60	12.04 ± 0.24	11.6	35.5 ± 22.4
40-day old, hGH-DNA	3	10.79 ± 0.60	13.19 ± 0.70	22.2	
40-day old, untreated scid	4	14.01 ± 0.31	15.27 ± 0.85	9.0	
80-day old, saline	4	12.16 ± 0.20	12.51 ± 0.25	2.9	25.6 ± 11.7
80-day old, hGH-DNA	3	12.17 ± 0.50	13.59 ± 0.50	11.7	
80-day old, untreated scid	5	15.04 ± 0.38	16.72 ± 0.40	11.2	
Tail length (cm)					
40-day old, saline	4	5.13 ± 0.20	5.80 ± 0.18	13.1	39.3 ± 18.2
40-day old, hGH-DNA	3	5.13 ± 0.20	6.37 ± 0.30	24.2	
40-day old, untreated scid	4	6.86 ± 0.10	7.26 ± 0.77	5.8	
80-day old, saline	4	5.97 ± 0.20	6.03 ± 0.18	1.0	22.8 ± 9.7
80-day old, hGH-DNA	3	5.83 ± 0.20	6.53 ± 0.20	12.0	
80-day old, untreated scid	5	7.32 ± 0.16	8.15 ± 0.17	11.3	
Femur length (mm)[1]					
40-day old, saline	4	–	9.96 ± 0.70	–	76.2 ± 36.4
40-day old, hGH-DNA	3	–	11.82 ± 0.85	18.7[2]	
40-day old, untreated scid	4	–	12.36 ± 0.71	–	
80-day old, saline	4	–	10.00 ± 0.70	–	39.9 ± 10.4
80-day old, hGH-DNA	4	–	11.61 ± 0.40	16.1[2]	
80-day old, untreated scid	6	–	14.04 ± 0.51	–	

Integrated statistical analysis based on catch-up growth for nose-to-tail length/tail length/right femur length/left femur length/mIGF1 concentration

40-day old mice			80-day old mice			P-value
Average ± SD	CV (%)	n	Average ± SD	CV (%)	n	
58.91 ± 31.20	52.9	17	27.98 ± 15.33	54.8	23	0.0002[1]

[1]Excluding IGF1 concentration P = 0.0129.
[1]Considering the average length of right and left femur.
[2]Femur length percent of increase is calculated with basis on saline-treated co-aged mice. All other percentages of increase are calculated with basis on initial values.

Table 4
Plasma mIGF1 and hGH concentrations for lit/scid mice during Bioassay III based on tibialis cranialis hGH plasmid administrations in comparison with co-aged scid mice.

Time after administration (days)	40 days old at time of administration								80 days old at time of administration					
	Saline in lit/scid			hGH-DNA in lit/scid			Co-aged scid		hGH-DNA in lit/scid			Co-aged scid		
	mIGF1 (ng/mL)	hGH	n	mIGF1 (ng/mL)	hGH	n	mIGF1 (ng/mL)	n	mIGF1 (ng/mL)	hGH	n	mIGF1 (ng/mL)	n	
15	58.0 ± 0.0	–	2	250.0 ± 42.4[1,2]	–	2	277.5 ± 127.3[1]	6	74.7 ± 56.7[2]	–	3	387.9 ± 36.8	6	
60	39.5 ± 17.1	1.49 ± 1.18	4	215.0 ± 48.2	6.83 ± 1.61	3	403.40 ± 40.4	4	160.0 ± 39.4	6.67 ± 1.15	4	458.0 ± 35.7	6	

[1] Non-significantly different (P > 0.05);
[2] Significantly different (P < 0.05).

The positive correlation found in non-dwarf mice between mIGF1 levels and age confirmed the pioneering data from the Jackson Laboratories on lit/+ mice, in which mIGF1 rose from ~100 ng/mL at about 1 week to a maximum (~550 ng/mL) only at 8 weeks of age [36]. In addition, Bioassay III provided the opportunity to test the efficiency of a recently developed less invasive method based on tibialis cranialis administration to both muscles, without exposition.

Parameters based on longitudinal growth, such as nose-to-tail, tail and femur lengths, have proven to be quite useful for accurately following the treatment process. The highest precision/lowest variation was found for the measurements of nose-to-tail and tail lengths, while the most variable parameter was body weight. These variations are clearly a consequence of at least two factors: (i) error related to the measurement procedure and (ii) individual physiological variations in each parameter. Femur measurements, in particular, showed a great sensitivity, especially considering that this bone does not undergo any significant increase in postpubertal dwarf mice. A highly significant difference ($P < 0.0001$) in catch-up growth was found between femur and all other physical measurements, confirming remarkable osteogenic effects of GH (see Table 1). The calculation of femur increase was however based not on initial individual femur length measurements, but rather on average values obtained from co-aged mice and hence may incorporate a considerable error. Initial and final x-ray measurements of bone length on the individual mice will certainly improve the accuracy, as we experienced in a recent methodological procedure (Cecchi et al., manuscript in preparation). The tail length was maintained as a separate parameter since it is the only one that, according to the literature, does not show any gender difference within either the lit/lit or lit/+ mice genotypes [36].

Young rodents were found to be particularly favorable recipients of plasmid-based intramuscular gene transfer. A better DNA delivery throughout the young muscle may be due to a lower content of extracellular matrix, with a consequent better distribution through the myofibers, to a greater propensity for cellular DNA uptake or even to an increased transcriptional activity [2,37–39]. Other authors have reported, however, that direct intramuscular DNA injection is affected by the age and gender of mice only in the absence of electroporation [40–41]. Our data appear to confirm these reports since younger treated animals did not show higher serum hGH concentrations; therefore, a different mechanism should be responsible for the increased mIGF1 levels. On the other hand, many laboratory and auxological parameters have shown that the age of onset for GHD treatment in children is inversely correlated with growth responses and that every effort should be made to enhance growth before puberty [42–44].

In conclusion, compared to previous investigations carried out by our research group employing a more invasive methodology [14–16], considerably higher catch-up growth and mIGF1 concentrations were obtained in the present study, with normal levels of mIGF1 being obtained in one particular situation of Bioassay III. This improvement was especially due to the use of younger animals that experienced higher IGF1 and better growth recovery. We do not exclude, however, that higher GH circulating levels may be needed for a completely successful preclinical treatment scheme in dwarf animals, also considering a future translation to clinic. Sagatio and co-workers [25] successfully treated a different model of isolated GHD by injecting double stranded AAV vectors into 10-day old mice; while higher at the beginning, at the end of a 6-month assay their mice presented GH serum levels comparable to ours. Although several difficulties must still be resolved for an adequate and correct DNA administration in such very young dwarf animals, we are confident that, by initiating a longer GH-DNA treatment on pubertal mice, a much better and possibly complete phenotypic correction can be achieved.

Table 5
Significance of the different growth parameters in untreated postpubertal (2–9 months of age) lit/scid or scid mice.

Parameter	n	Slope of growth curve (g, cm or mm/month)	Correlation coefficient (r)	Significance of the correlation curve
Body weight (g)				
lit/scid	39	0.110	0.268	NS
scid	39	1.142	0.544	P < 0.001
Nose-to-tail length (cm)				
lit/scid	39	0.132	0.422	P < 0.01
scid	39	0.505	0.714	P < 0.001
Tail length (cm)				
lit/scid	39	0.049	0.332	P < 0.05
scid	39	0.290	0.695	P < 0.001
Right femur (mm)				
lit/scid	39	0.035	0.117	NS
scid	35	0.540	0.715	P < 0.001
Left femur (mm)				
lit/scid	39	0.024	0.084	NS
scid	37	0.489	0.703	P < 0.001

Conflict of interest

The authors declare that there is no conflict of interest.

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