Growth Hormone & IGF Research 20 (2010) 118–126

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

# Ames dwarf (*Prop1*$^{df}$/*Prop1*$^{df}$) mice display increased sensitivity of the major GH-signaling pathways in liver and skeletal muscle

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ARTICLE INFO

*Article history:*
Received 18 November 2008
Revised 10 November 2009
Accepted 11 November 2009
Available online 21 December 2009

*Keywords:*
Ames dwarf mice
GH-signal transduction
GH
STAT
Erk
Akt

ABSTRACT

*Context:* Growth hormone (GH) is an anabolic hormone that regulates growth and metabolism. Ames dwarf mice are natural mutants for *Prop1*, with impaired development of anterior pituitary and undetectable levels of circulating GH, prolactin and TSH. They constitute an endocrine model of life-long GH-deficiency. The main signaling cascades activated by GH binding to its receptor are the JAK2/STATs, PI-3K/Akt and the MAPK Erk1/2 pathways.
*Objectives:* We have previously reported that GH-induced STAT5 activation was higher in Ames dwarf mice liver compared to non-dwarf controls. The aim of this study was to evaluate the principal components of the main GH-signaling pathways under GH-deficiency in liver and skeletal muscle, another GH-target tissue.
*Methods:* Ames dwarf mice and their non-dwarf siblings were assessed. Animals were injected *i.p.* with GH or saline 15 min before tissue removal. Protein content and phosphorylation of signaling mediators were determined by immunoblotting of tissue solubilizates.
*Results:* GH was able to induce STAT5 and STAT3 tyrosine phosphorylation in both liver and muscle, but the response was higher for Ames dwarf mice than for non-dwarf controls. When Erk1/2 activation was assessed in liver, only dwarf mice showed GH-induced phosphorylation, while in muscle no response to the hormone was found in either genotype. GH-induced Akt phosphorylation at Ser473 in liver was only detected in dwarf mice. In skeletal muscle, both normal and dwarf mice responded to a GH stimulus, although dwarf mice presented higher GH activation levels. The phosphorylation of GSK-3, a substrate of Akt, increased upon hormone stimulation only in dwarf mice in both tissues. In contrast, no differences in the phosphorylation of mTOR, another substrate of Akt, were observed after GH stimulus, either in normal or dwarf mice in liver, while we were unable to determine mTOR in muscle. Protein content of GH-receptor and of the signaling mediators studied did not vary between normal and dwarf animals in the assessed tissues.
*Conclusion:* These results show that several components of the main GH-signaling pathways exhibit enhanced sensitivity to the hormone in liver and muscle of Ames dwarf mice.


## 1. Introduction

Growth hormone (GH) participates in many metabolic processes beyond longitudinal growth promotion, including protein synthesis, carbohydrate and lipid metabolism. At the cellular level, GH promotes proliferation, differentiation and survival and motility. To exert its actions, GH must first bind to its specific receptor, the GHR. Ligand binding confers the proper conformation to a preformed inactive receptor dimer, resulting in the activation of the associated Janus kinase JAK2 [1,2]. This kinase, in turn, activates several intracellular mediators leading to different signaling pathways. The main GH-signaling pathway comprises STAT (signal transducers and activators of transcription) proteins; GH has been shown to activate STAT1, STAT3 and STAT5a/b. Two other major pathways induced by GH are the phosphatidylinositol-3′kinase (PI-3K)/protein kinase B (PKB/Akt) and the mitogen-activated protein (MAP)-kinase pathways [1,3,4]. Even when GH-signaling events largely rely on JAK2 activation, additional pathways, independent of JAK2, have also been described [5,6].

Ames dwarf mice are homozygous for a spontaneous mutation in the prophet of pituitary factor-1 (*Prop1*) gene leading to a primary deficiency of GH, prolactin (PRL) and thyroid-stimulating hormone (TSH) and a secondary suppression of hepatic expression

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doi:10.1016/j.ghir.2009.11.003

of insulin-like growth factor (IGF)-I and circulating IGF-I levels [7,8]. Although they exhibit normal body size at birth, postnatal growth is greatly reduced and adult body size is 30–50% of normal values. These animals also present alterations in body composition, sexual function and carbohydrate metabolism, as well as hypothyroidism and remarkably extended longevity [9,10]. GH treatment causes plasma IGF-I levels and body weight increase in dwarf mice, along with other physiological and biochemical parameters, indicating that the animals are able to respond to the hormone [11–13].

In a previous work we have reported that GH-induced STAT5 activation was enhanced in dwarf mice liver, suggesting that these mice have increased sensitivity to GH in this tissue [14]. However, GH exerts its actions on many other organs and tissues; moreover, GH also triggers other signaling pathways beyond the JAK2/STAT5 cascade. The purpose of this study was to determine if the higher GH sensitivity found in Ames mice liver for the JAK2/STAT5 pathway applies also to other signaling pathways activated upon GH stimulation, and whether the effects of this mutation on GH-signaling found in liver are paralleled in muscle, which is also a major target tissue of GH action.

## 2. Materials and methods

### 2.1. Chemicals

Ovine GH (oGH) was obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA. Polyvinylidene difluoride (PVDF) membranes, ECL-Plus and Hyperfilm-ECL were from Amersham Biosciences (Piscataway, NY, USA). Antibody anti-phospho-STAT5a/b Tyr694/696 was from Upstate Laboratories (Lake Placid, NY, USA). Antibodies anti-phospho-Akt Ser473, anti-Akt, anti-phospho-GSK-3β Ser9, anti-GSK-3β, anti-phospho-mTOR Ser2448, anti-mTOR, anti-phospho-p44/42 MAP kinase Thr202/Tyr204, anti-p44/42 MAP kinase, and anti-phospho-STAT3 Tyr705 were from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibody anti-STAT3 was purchased from Transduction Laboratories (Lexington, KY, USA); anti-STAT5 and secondary antibodies conjugated to horse radish peroxidase (HRP) were from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA). Antibody anti-$GHR_{cyt\text{-}AL47}$ was kindly provided by Frank (University of Alabama at Birmingham, Birmingham, Alabama 35294-0012, USA) and has been previously described [15]. All other chemicals were of reagent grade.

### 2.2. Animals

Ames dwarf mice ($Prop1^{df}/Prop1^{df}$) and normal (+/+ or +/$Prop1^{df}$) littermates, 6–9 months of age were used. Mice were produced in a closed colony with a heterogeneous genetic background. Normal littermates were used as controls; we are not aware of any evidence that animals heterozygous for Ames dwarfism may differ from homozygous normal animals. Mice were housed in groups 4–5 per cage in plastic "shoe box type" cages with wood chips in a room with controlled photoperiod of 12 h light:12 h dark cycle (lights on from 06:00 to 18:00 h) and a temperature of 22 ± 2 °C. Each cage was equipped with individual filter top (microisolator unit). Sentinel animals were housed in the same room and used for testing for antibodies to all major murine pathogens. The results of these tests were uniformly negative. Animals were given free access to a nutritionally balanced diet (Rodent Laboratory Chow 5001; not autoclaved; 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet, PMI Feeds, Inc., St. Louis, MO, USA) and tap water. The appropriateness of the experimental procedure, the required number of animals used, and the method of acquisition were in compliance with federal and local laws, and approved by the Southern Illinois University Animal Care and Use Committee.

### 2.3. Hormone treatment

The mice were fasted overnight prior to hormone stimulation. 1.8 mg of oGH per kg of body weight (BW) in 0.2 ml 0.9% NaCl were injected *i.p.* To evaluate basal conditions, mice were injected with saline. Hormone dose and stimulus duration were chosen to activate the different signaling mediators to be studied. Mice were killed 15 min after GH injection; the tissues were removed and kept frozen at −80 °C until use.

### 2.4. Preparation of tissue extracts

The liver and skeletal muscle (upper hind limb mixed muscles, mainly quadriceps femoris) were homogenized in 1% Triton, 100 mM Hepes, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 0.035 trypsin inhibitory units/ml aprotinin (pH 7.4) at 4 °C. Tissue homogenates were centrifuged at 100,000*g* for 30 min at 4 °C to remove insoluble material. Protein concentration of supernatants was determined by the method of Bradford [16]. An aliquot of solubilized tissues was diluted in Laemmli buffer, boiled for 5 min and stored at −20 °C until electrophoresis.

### 2.5. Immunoblotting

Samples were subjected to electrophoresis in SDS–polyacrylamide gels using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Equal amount of total protein was loaded in each lane. Electrotransference of proteins from gel to PVDF membranes was performed for 1 h at 100 V (constant) using the Bio-Rad miniature transfer apparatus in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. To reduce non-specific antibody binding, membranes were incubated for 2 h at room temperature in T-TBS blocking buffer (10 mM Tris–HCl, 150 mM NaCl, and 0.02% Tween 20, pH 7.6) containing 3% BSA. The membranes were then incubated overnight at 4 °C with the primary antibodies: anti-phospho-STAT5a/b Tyr694/696 (1:1000), anti-STAT5 (1:4000), anti-phospho-Akt Ser473 (1:1000), anti-Akt (1:1000), anti-phospho-GSK-3β Ser9 (1:1000), anti-GSK-3β 1:2000, anti-phospho-mTOR Ser2448 (1:1000), anti-mTOR (1:1000), anti-phospho-p44/42 MAP kinase Thr202/Tyr204 (1:1000), anti-p44/42 MAP kinase (1:1000), anti-phospho-STAT3 Tyr705 (1:1000), anti-STAT3 (1:4000) and anti-GHR (1:1000).

After washing with T-TBS, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature and washed in T-TBS. Blots were visualized by the enhanced chemiluminiscence (ECL-Plus, Amersham Biosciences, Piscataway, NY, USA) method, and exposed using Hyperfilm-ECL. Images were captured using a digital imaging system (Foto-analyst Investigator, Fotodyne Inc., Hartland, WI, USA) and band intensity was quantified using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA). Results are expressed as a percentage of the mean value measured for the group presenting the higher signal, which was the dwarf GH-stimulated group in every case.

To evaluate the protein content of the different signaling molecules on the same membranes where their phosphorylation status was assessed, the membranes were reprobed. Membranes were stripped by washing with acetonitrile for 10 min and then incubated in stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris/HCl, pH 6.7) for 40 min at 50 °C under shaking, washed with deionized water and blocked with 3% BSA in T-TBS, prior to incubation with the respective antibody.

### 2.6. Statistical analysis

Experiments were performed analyzing all groups of animals in parallel, *n* representing the number of different individuals used in each group. Results are presented as mean ± standard error (SE) of the number of samples indicated. Statistical analyses were performed by ANOVA followed by the Newman–Keuls multiple comparison test using the GraphPad Prism 4 statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Data were considered significantly different if $p < 0.05$.

## 3. Results

### 3.1. STAT5 and STAT3 phosphorylation and protein content

Growth hormone promotes the phosphorylation of two STAT5 proteins, STAT5a at Tyr694, and STAT5b at Tyr696, but since anti-phospho-tyrosine specific antibodies and anti-total-protein antibodies do not discriminate between them, they are collectively referred to as STAT5. Growth hormone stimulation induced a marked increase in STAT5 phosphorylation in liver and skeletal muscle both in normal and in dwarf Ames mice. However, the level of STAT5 tyrosine phosphorylation obtained upon GH stimulation was higher for dwarf mice compared to their normal siblings, while no significant difference was observed in the basal phosphorylation levels between normal and dwarf mice (Fig. 1A and C). In both liver and muscle, STAT5 protein content was similar in normal and dwarf animals and it was not altered by GH treatment (Fig. 1B and D).

When STAT3 phosphorylation at Tyr705 was assessed, results were similar to those observed for STAT5. GH-induced phosphorylation of STAT3 in dwarf mice was significantly higher than in normal mice, both in liver and in muscle (Fig. 2A and C). No significant differences were observed in the basal phosphorylation and in the protein content levels between normal and dwarf mice in either tissue (Fig. 2A–D).

### 3.2. Erk1/2 phosphorylation and protein content

The MAP kinases Erk1/2 (p44-42 MAPK) are activated by phosphorylation at Thr202 and Tyr204, and this dual phosphorylation pattern can be recognized by specific antibodies. In liver of dwarf mice there was a significant increase in Erk1/2 phosphorylation level upon GH stimulation; however, identical GH treatment failed to induce phosphorylation of Erk1/2 in normal mice (Fig. 3A). In contrast, in the muscle, GH treatment did not produce any change in the phosphorylation status of Erk1/2, in either normal or dwarf mice (Fig. 3C). Basal phosphorylation and protein content levels did not differ significantly in normal and in dwarf mice in both tissues studied (Fig. 3A–D). In muscle, pErk1 exhibited slightly higher basal phosphorylation levels in some dwarf mice, although this was not seen consistently (Supplementary Fig. 1).

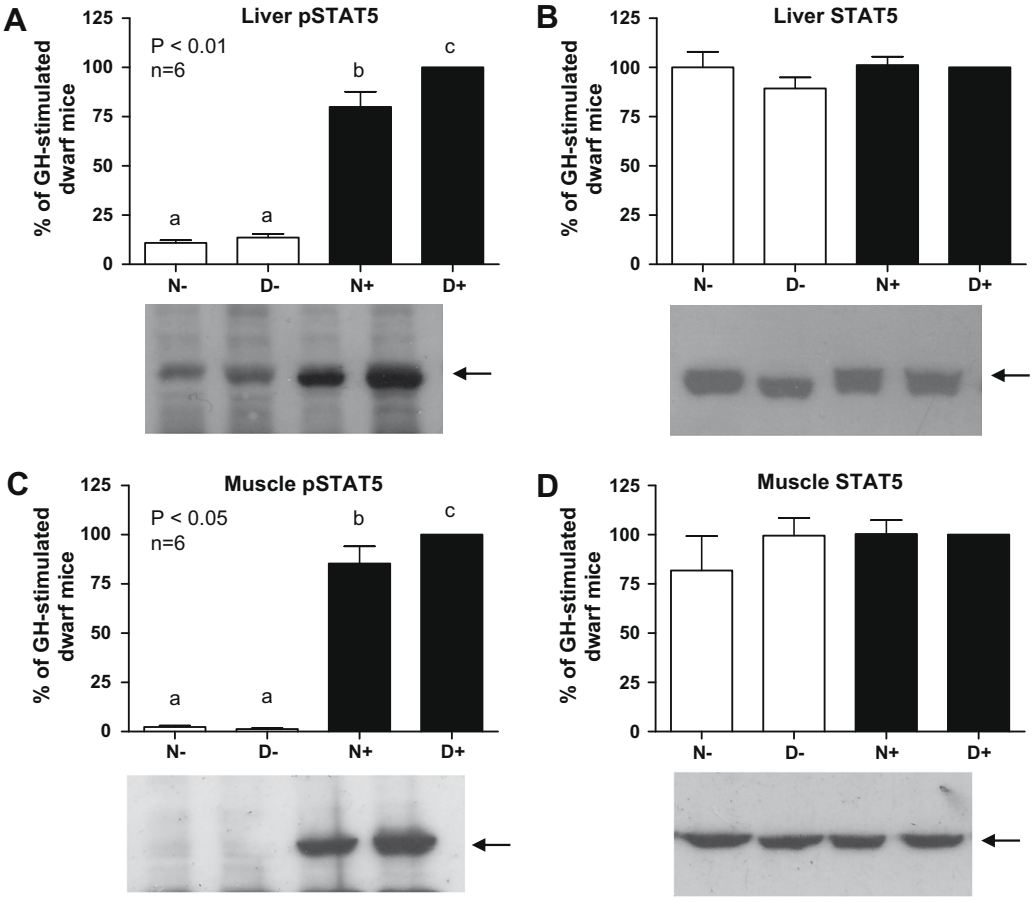


**Fig. 1.** Liver and muscle STAT5 Tyr694/696 phosphorylation status and protein content. Sixty micrograms of total protein from liver or muscle extracts from normal (N) and dwarf (D) mice treated with GH (+) or saline (−) were separated by SDS–PAGE and subjected to immuno-blot analysis with anti-phospho-specific antibody (A and C); the membranes were then reprobed with anti-specific protein antibody to assess protein content (B and D). Protein phosphorylation was expressed as a percentage of the mean value measured for GH-stimulated dwarf mice. Data are the mean ± SEM of six samples per group, each one representing a different animal, run in two separate experiments (three sets per experiment). Different letters denote significant difference at $p < 0.05$. The arrows indicate the specific bands that were quantified.

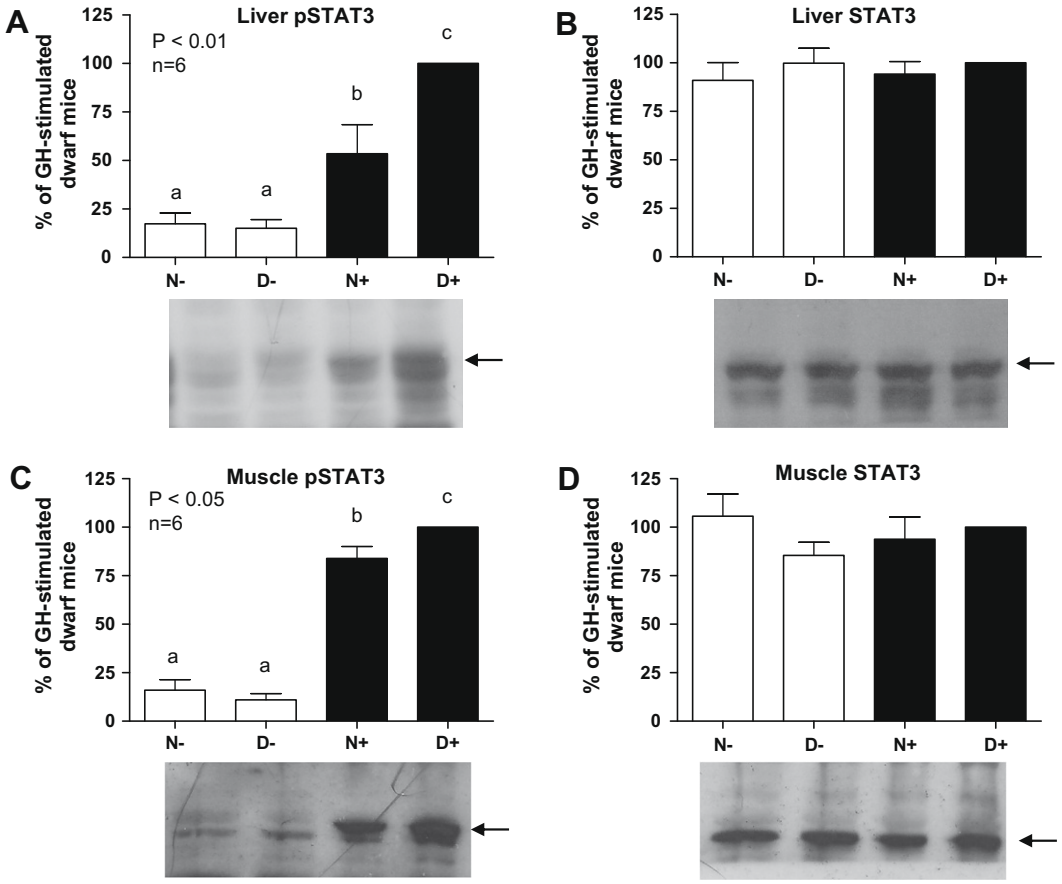


**Fig. 2.** Liver and muscle STAT3 Tyr705 phosphorylation status and protein content. See Fig. 1 for description.

### 3.3. Akt phosphorylation and protein content

Akt is a serine/threonine kinase activated by phosphorylation at Thr308 and Ser473, with the latter conferring maximal activity to the kinase. Akt phosphorylation at Ser473 displayed differential outcome of GH treatment with genotype in the liver as compared to the muscle. In liver, GH induced Akt phosphorylation in dwarf mice but not in normal animals (Fig. 4A). In muscle, GH stimulation produced an increase in the phosphorylation of Akt in normal and in dwarf mice, although the level of phosphorylation was higher in dwarf than in normal animals (Fig. 4C). Basal levels of phosphorylated Akt did not significantly differ between normal and dwarf mice (Fig. 4A and C). Akt protein content was similar in normal and dwarf mice and did not vary with GH treatment in either of the tissues studied (Fig. 4B and D).

### 3.4. GSK-3 and mTOR phosphorylation and protein content

In order to evaluate if the increased Akt phosphorylation in response to GH observed in dwarf mice resulted in a higher activation of downstream effectors, and to associate these observations with previous results obtained for Ames dwarf mice [17], we evaluated the phosphorylation of glycogen synthase kinase-3 (GSK-3) and mammalian target of rapamycin (mTOR), which are serine/threonine protein kinases downstream from PI-3K/Akt signaling pathway.

Two major isoforms of GSK-3 have been described: α and β. The activity of GSK-3 is inhibited by phosphorylation of Ser9 in GSK-3β or Ser21 in GSK-3α; several kinases can phosphorylate these residues, including Akt [18,19]. We determined the phosphorylation of GSK-3β on Ser9 in liver and muscle and we were unable to detect GH-induced phosphorylation of this residue in normal mice. However, dwarf mice showed a significant response to GH stimulation in both tissues (Fig. 5A and C). The protein content of this protein did not change in any of the analyzed groups (Fig. 5B and D). Although the antibodies used were against isoform β, two bands were detected in liver, most probably due to cross-reactivity with isoform α. Both bands exhibited a parallel pattern and were analyzed together.

Akt may activate mTOR indirectly by phosphorylating proteins involved in the regulation of mTOR activity. In addition, mTOR is directly phosphorylated by Akt at Ser2448, but the relevance of this phosphorylation in its activity is not clear [20]. Moreover, several Akt-independent mechanisms for mTOR regulation have been described (reviewed by Memmott and Dennis, 2009) [20]. In the liver, mTOR phosphorylation at Ser2448 did not significantly change between normal and dwarf mice, either at the basal level or after GH stimulation (Fig. 6A). Similar mTOR protein content was observed in liver in the different animal groups (Fig. 6B), while we were unable to detect mTOR in skeletal muscle (Supplementary Fig. 2). Both for mTOR phosphorylation and protein content, two immunoreactive bands were detected and they were quantified together.

### 3.5. GHR protein content

To assess if the described effects on GH-signaling are associated with changes in GHR expression, we determined the protein content of the receptor in liver and muscle. Similar GHR protein levels were observed in normal and dwarf mice in both tissues (Fig. 7). In dwarf mice a tendency to diminished basal GHR levels compared to normal controls was observed in the liver, although

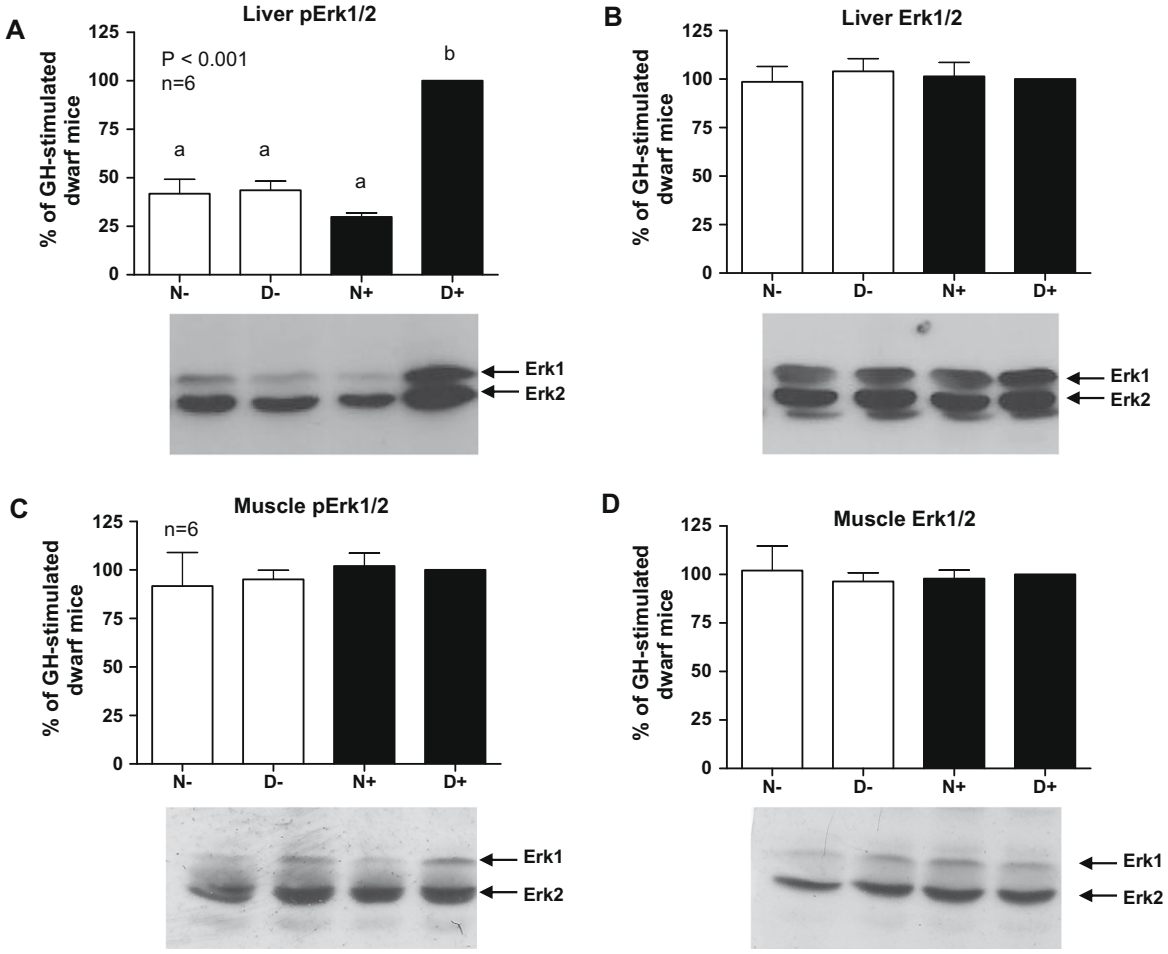


**Fig. 3.** Liver and muscle Erk1/2 Thr202/Tyr204 phosphorylation status and protein content. See Fig. 1 for description. Each column represents the sum of Erk1 and Erk2 values, because the pattern observed for both bands was similar.

it did not reach statistical significance. In muscle, the abundance of GHR was markedly lower than in liver, making the accurate quantification of the band difficult, as in some samples it was almost undetectable.

## 4. Discussion

In this study we have assessed GH sensitivity in two major GH targets: the liver and the skeletal muscle of Ames dwarf mice and their control littermates. Activation of the principal pathways induced by GH was evaluated and hypersensitivity to the hormone was found in GH-deficient mice in both tissues.

Endocrine mutants constitute unique models to evaluate hormone function and to elucidate its molecular mechanisms of action. In this study, *Prop1*$^{df}$/*Prop1*$^{df}$ (Ames dwarf) mice were used to evaluate the effects of congenital GH-deficiency on GH-signaling. This mutant is characterized by the lack of prophet of pituitary factor-1 (Prop1), an early transcription factor required for proper pituitary development, leading to the absence or extreme reduction of anterior pituitary cells that produce GH, PRL and TSH [7,8]. Appraisal of GH-signaling in these animals is of particular interest because the reduction in the GH/IGF-I signaling pathways has been associated with markedly increased life-expectancy (reviewed by Bartke and Brown-Borg [9], Bartke [21] and Berryman et al. [22]). Moreover, alterations in the regulation of the intracellular cascades elicited by GH have been related to impaired cellular proliferation.

Signal transducers and activators of transcription are the most prominent GH-signaling mediators. STAT5b is the principal factor activated by growth hormone in GH-target tissues; it has been shown to be related to growth promotion, as evidenced by the phenotype of STAT5b knock-out mice [23–25]. Moreover, STAT5b, the STAT5 predominantly expressed in liver, has been related to IGF-I gene expression in this tissue [26,27]. We have previously shown that Ames dwarf mice exhibit diminished or unaffected hepatic GH-receptor levels, with unaltered hepatic content of JAK2 and STAT5a/b proteins [28,14]. Furthermore, in dwarf mice, STAT5 became phosphorylated upon GH stimulation with lower hormone doses than those that were required to increase phosphorylation in control siblings. This correlated with diminished levels of the suppressor of cytokine signaling CIS, which in Ames dwarfs were 20% of those found in normal mice liver [14]. In this study we have extended our observations to other GH-signaling mediators and, in addition, to another GH-effector tissue, the skeletal muscle. In agreement with our previous report, hepatic STAT5 phosphorylation upon GH stimulation was greater in Ames dwarf than in non-dwarf siblings used as controls, and a similar difference was found in the muscle. This higher response was not associated with changes in STAT5 protein content, indicating that the hypersensitivity to the hormone reflects a higher phosphorylation capacity rather than upregulated STAT5 protein expression levels. Another transcription factor of this family activated by GH is STAT3, which plays an important role in promoting cell-cycle progression and cellular transformation, and in preventing apoptosis. It is critical

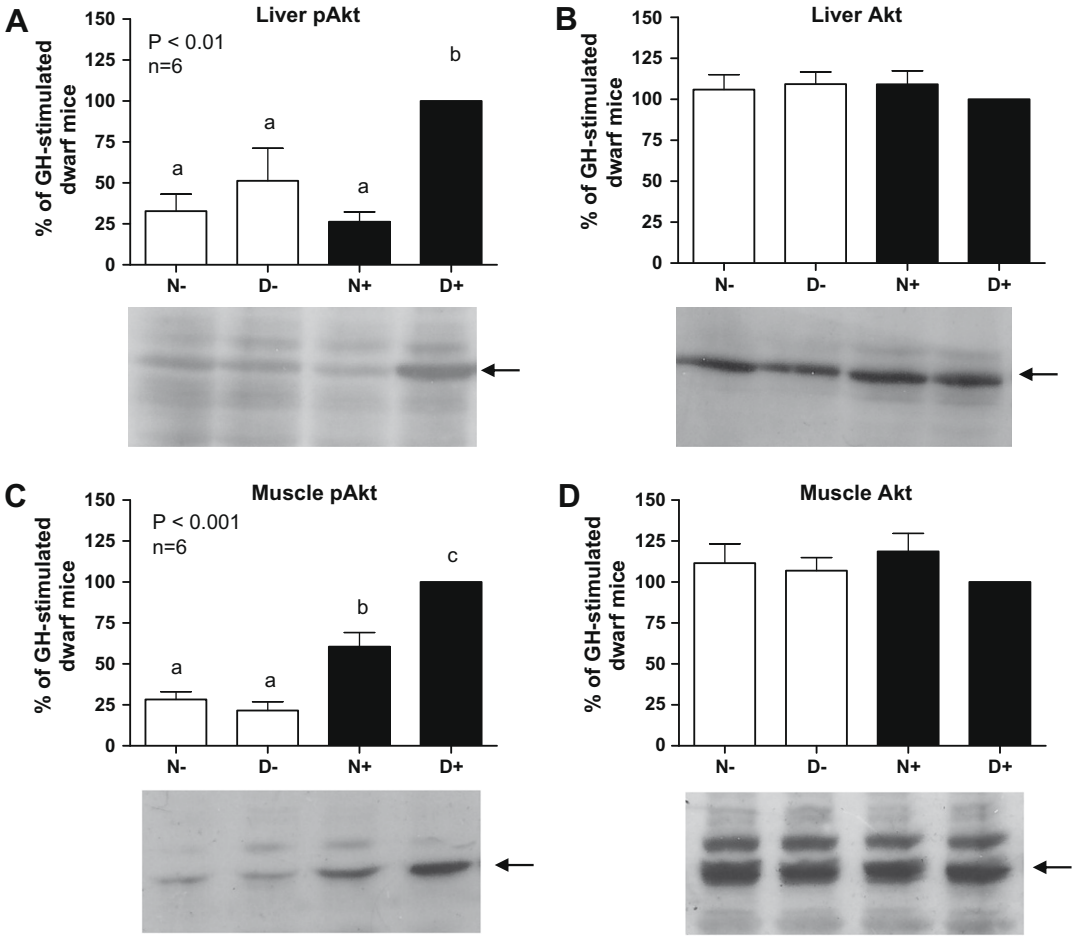


**Fig. 4.** Liver and muscle Akt Ser473 phosphorylation status and protein content. See Fig. 1 for description.

during embryogenesis since STAT3-knock-out mice die before reaching gastrulation [29]. When GH-induced STAT3 activation was evaluated, a similar pattern to that of STAT5 was observed, although the difference between normal and dwarf was more pronounced in liver than in muscle.

The mitogen-activated protein kinase (MAPK) cascade participates in the control of cell proliferation, differentiation and migration. Extracellular signal-regulated kinases 1 and 2 (Erk1/2) are isoforms of the classical MAPKs that can be activated by a variety of mitogens and growth factors [30]. GH activates Erk1/2 in different models [3,4,31]. However, we did not detect hepatic Erk1/2 phosphorylation upon GH stimulus in normal mice, in agreement with our previous report [32]. Importantly, under identical conditions, dwarf mice did respond to the hormone. This would suggest that Erk may not be activated in the liver by GH under physiological conditions, but responds to an acute GH stimulus in the setting of congenital GH-deficiency. This observation cannot be extended to muscle, where neither normal nor dwarf mouse tissue responded to the hormone, suggesting this GH-signaling pathway may not be relevant in this tissue.

Growth hormone may activate the PI-3K pathway by JAK2 phosphorylation of IRSs (insulin receptor substrates), which leads to their association with the p85 subunit of PI-3K, whereas other possible mechanisms that may involve CrkII-IRS-1 interaction or direct binding of p85 subunit of PI-3K to the GHR have been suggested (reviewed by Lanning and Carter-Su [1]). Akt/PKB is a serine/threonine kinase downstream of PI-3K, which is a crucial regulator of several cellular processes including cell survival, proliferation and metabolism [18]. In liver and muscle of Ames dwarf mice, Akt can be activated by growth factors such as insulin [33,34] and GH (present results). However, in liver of normal mice we did not find GH-induced activation of Akt, contrary to observations in H4IIE hepatoma cells [4,6,35], suggesting that this protein is not a major signaling mediator activated by GH in this tissue *in vivo*. Under the same conditions, however, activation response was observed in muscle of normal controls, in line with the well-characterized role of Akt as a mediator of muscle hypertrophy [36,37].

Basal Akt phosphorylation values found in the liver and skeletal muscle of Ames dwarf mice were similar to those of control mice. These results are in accordance with previous work from our laboratory, in which Akt basal phosphorylation levels, Akt protein content and PI-3K basal activity were not significantly different between normal and dwarf Ames mice, either in liver or in muscle [33,34]. These observations do not coincide with those found for Snell dwarf mice with a spontaneous mutation on *Pit-1* that results in the same combined pituitary hormone deficiency as Ames dwarf mice. Snell dwarf mice had decreased hepatic Akt protein levels and basal phosphorylation compared to heterozygote siblings [38]. The most likely reason for the differences between Ames and Snell dwarfs is different genetic backgrounds, which can have an important impact on phenotype [38]. The activation status of downstream effectors of the PI-3K/Akt pathway related to protein synthesis including mTOR and translation initiation factors was also reported to be diminished in Snell dwarf mice [38]. Similarly, in Ames dwarfs, down-regulation of downstream translation regulatory proteins was reported in liver and in gastrocnemius skeletal

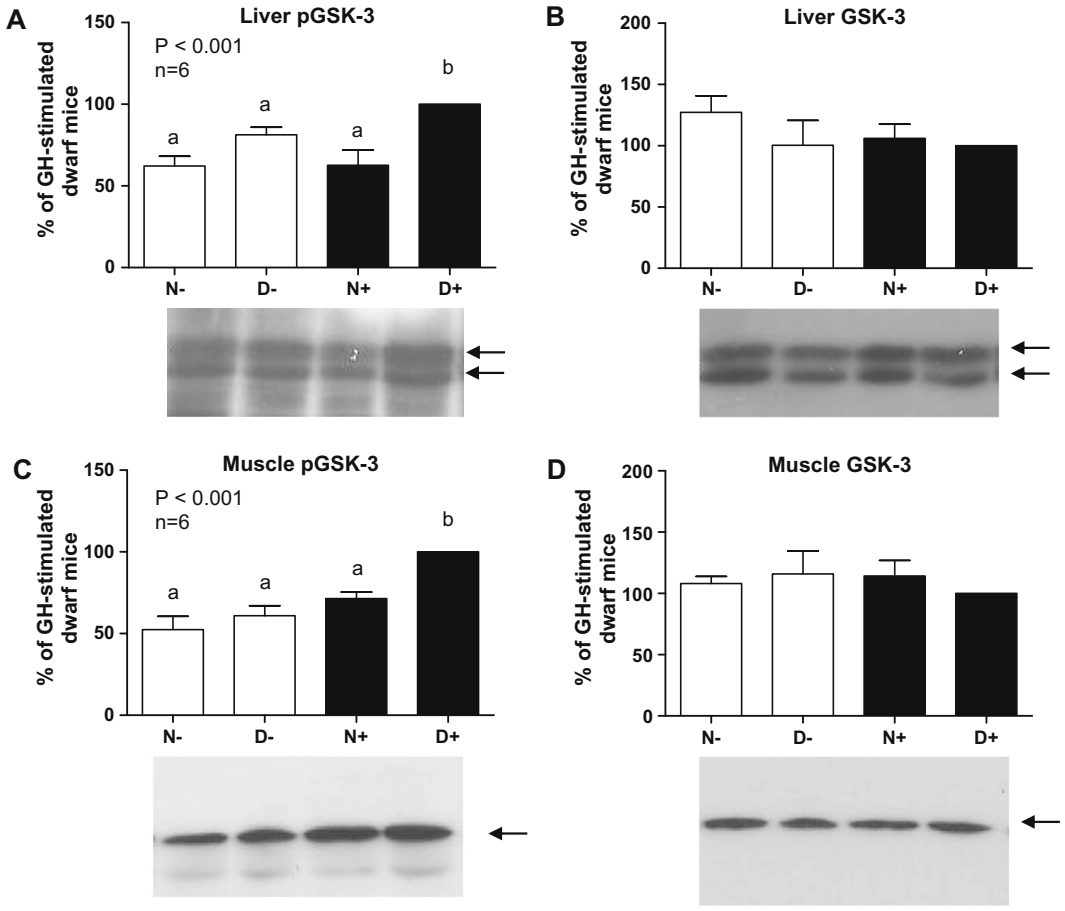


**Fig. 5.** Liver and muscle GSK-3β Ser9 phosphorylation status and protein content. See Fig. 1 for description.

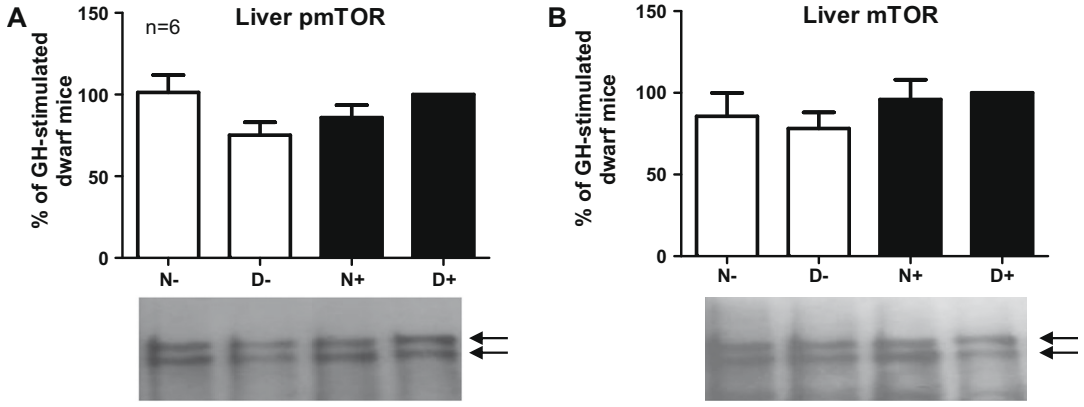


**Fig. 6.** Liver mTOR Ser2448 phosphorylation status and protein content. Sixty micrograms of total protein from liver extracts from normal (N) and dwarf (D) mice treated with GH (+) or saline (−) were separated by SDS–PAGE and subjected to immuno-blot analysis with anti-phospho-specific antibody (A); the membranes were then reprobed with anti-specific protein antibody to assess protein content (B). Protein phosphorylation was expressed as a percentage of the mean value measured for GH-stimulated dwarf mice. Data are the mean ± SEM of six samples per group, each one representing a different animal, run in two separate experiments (three sets per experiment). Non-significant differences were observed. The arrows indicate the specific bands that were quantified.

muscle [17]. As we detect no alterations in the basal phosphorylation of Akt in Ames dwarf mice, the down-regulation of downstream components of the PI-3K/Akt/mTOR reported in these mice [17] may not be a direct consequence of diminished Akt phosphorylation.

To further evaluate Akt signaling we assessed the phosphorylation status of two of its downstream targets, GSK-3 and mTOR. GSK-3 is involved in the regulation of many cellular processes including proliferation, mobility, differentiation and apoptosis and it has been linked to several diseases [18,19]. Dwarf mice showed a significant increase in the phosphorylation of GSK-3 in liver and in skeletal muscle in response to GH stimulation, while GH-induced phosphorylation at this residue was not detected in normal mice. GH has been recently shown to activate mTOR and downstream components of the translational machinery, as well as protein synthesis *per se*, in hepatoma cells [35]. In contrast to re-

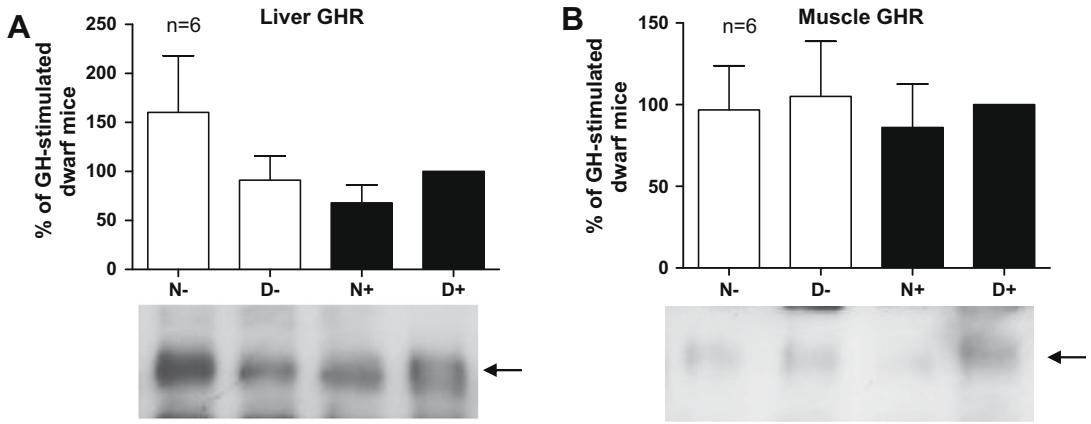


**Fig. 7.** Liver and muscle GHR protein content. Sixty micrograms of total protein from liver or muscle extracts from normal (N) and dwarf (D) mice treated with GH (+) or saline (−) were separated by SDS–PAGE and subjected to immuno-blot analysis with anti-GHR antibody. Protein content was expressed as a percentage of the mean value measured for GH-stimulated dwarf mice. Data are the mean ± SEM of six samples per group, each one representing a different animal, run in two separate experiments (three sets per experiment). Non-significant differences were observed. The arrows indicate the specific bands that were quantified.

sults obtained for GSK-3, GH stimulation did not induce an increase in the phosphorylation of mTOR in the residue target of Akt, and we were unable to detect differences in the basal phosphorylation levels of this protein between normal and dwarf mice liver. Considering these results, one could speculate that there is a differential regulation of signaling pathways downstream Akt. Interestingly, results of *in vitro* kinase assays for Akt in Snell mice liver using two different substrates suggested differential downstream targeting by the kinase [38].

Liver has been the most studied GH-target tissue, mainly in the context of the GH regulation of IGF-I expression. However, GH-signaling in non-hepatic tissues has been less characterized. The skeletal muscle is another major site of GH action, since GH is associated with growth and muscle mass maintenance. Several studies have addressed GH-signaling pathways in muscle both *in vivo*, in rats [39,40] and *in vitro*, in C2C12 skeletal muscle cells [41,42]. The GHR/JAK2/STAT5a/b signaling pathway is readily activated in response to GH in muscle cells; moreover, the PI-3K and the MAPK pathways are also engaged. A transient activation of the Erks and Akt/PKB and increases in IGF-I mRNA expression have been reported in mouse C2C12 muscle cells in response to GH [41]. However, we did not find GH-induced Erk activation in mouse muscle. On the other hand, contrary to our observations, Akt was not activated by GH in human skeletal tissue after an intravenous bolus, while MAPK activation did not follow a GH-response pattern [43]. These results suggest that while STAT proteins are unequivocally activated in muscle in all the models studied, other pathways do not exhibit the same behavior, probably reflecting they are not major signaling pathways for GH in this tissue, or that their regulation is species-specific or more complex. Growth hormone stimulated the phosphorylation of STAT5 but not of IRS-1 and SHC in liver and in skeletal muscle of normal rats [40]. However, as PI-3K/Akt and MAPK signaling pathways are also activated by insulin, different physiological conditions that increase insulin levels may affect basal activation levels of these pathways, which could mask GH-response [39].

The three main signaling pathways activated by GH present enhanced sensitivity to the hormone in Ames dwarf mice in the tissues studied. Higher sensitivity to hormone stimulation could be secondary to increased receptor levels; however, as GH levels are known to positively correlate with GH-receptor expression in liver, diminished total GHR content could have been expected in a GH-deficiency model [44,28]. Therefore, we have determined the abundance of GHR in liver and in muscle but found no significant differences between normal and dwarf mice in either tissue. Diminished levels of CIS in liver could be responsible for the higher sensitivity found for the STAT5 signaling pathways studied in Ames dwarf mice [14]. Since CIS may negatively modulate GH-signal by acting as an adaptor protein that targets GHR for internalization and degradation, rather than by competing with STAT5 binding to phosphorylated GHR [45,46], decreased levels of CIS found in liver would affect not only STAT5 signaling but also the other pathways activated by GH. Unfortunately, we were not able to detect CIS in skeletal muscle, probably due to lower abundance of this protein in this tissue. Other mechanisms underlying increased sensitivity of the signaling mediators to the hormone, both in liver and muscle, remain to be elucidated.

GH-signaling mediators have been implicated in malignant transformation, and GH-overexpressing mice have higher incidence of liver neoplasia [47,48,21]. In contrast, GH-deficient mice have reduced occurrence of cancer [49,50]. Since GH overexpression can be associated with higher protein content of mediators related to cell proliferation and survival [32], decreased expression and/or basal activation of these mediators in liver of dwarf mice could have been expected. However, the relative abundance of these proteins does not seem to decline under GH-deficiency.

In summary, we show that GH-deficient Ames dwarf mice present GH-hypersensitivity in liver and muscle, since GH-signaling mediators of the JAK2/STAT, PI-3K/Akt and MAPK cascades have higher activation capacity than those measured in the corresponding normal animals in response to the same GH dose. This hypersensitivity apparently reflects altered regulation at a post-receptor level, because GH-receptor content is not increased in these animals. Elucidating how these mediators behave in the setting of chronically decreased levels of growth factors may help shed light on various physiological and pathological processes.

## Conflict of interest

None declared.

## Acknowledgements

We thank Dr. S.J. Frank for anti-GHR antibody. JGM, LG, DT, FPD and AIS are Career Investigators of CONICET, JFG is supported by a fellowship from UBA and MCM is supported by a Fellowship from CONICET. Support for these studies was provided by UBA, CONICET,

and ANPCYT (Argentina) to DT and AIS, and by NIH via grant AG 19899 and by the Ellison Medical Foundation to AB.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ghir.2009.11.003.

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