Transgenic mice overexpressing GH exhibit hepatic upregulation of GH-signaling mediators involved in cell proliferation

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Abstract

Chronically elevated levels of GH in GH-transgenic mice result in accelerated growth and increased adult body weight. We have previously described that the GH-induced JAK2/STAT5-signaling pathway is desensitized in the liver of transgenic mice overexpressing GH. However, these animals present increased circulating IGF-I levels, increased hepatic GHR expression, and liver organomegaly due to hypertrophy and hyperplasia, which frequently progress to hepatomas as the animals age, indicating that action of GH on the liver is not prevented. In the present study, we have evaluated other GH-signaling pathways that could be activated in the liver of GH-transgenic mice. Upon GH administration, normal mice showed an important increment in STAT3 phosphorylation level, but transgenic mice did not respond to acute GH stimulation. However, STAT3 was constitutively phosphorylated in transgenic mice, whereas its

protein content was not increased. GH-transgenic mice showed overexpression of c-Src, accompanied by an elevation of its activity. Other signaling mediators including focal adhesion kinase, epidermal growth factor receptor, Erk, Akt, and mammalian target of rapamycin displayed elevated protein and basal phosphorylation levels in these animals. Thus, GH-overexpressing transgenic mice exhibit hepatic upregulation of signaling mediators related to cell proliferation, survival, and migration. The upregulation of these proteins may represent GH-signaling pathways that are constitutively activated in the presence of dramatically elevated GH levels throughout life. These molecular alterations could be implicated in the pathological alterations observed in the liver of GH-transgenic mice.

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Introduction

Growth hormone (GH) is a major regulator of body growth and metabolism. It exerts its actions by binding to its membrane GH receptor (GHR), with the consequent activation of the receptor associated tyrosine kinase JAK2, which subsequently phosphorylates diverse signaling mediators (Frank 2001, Waters *et al.* 2006, Brooks *et al.* 2007, Lanning & Carter-Su 2007). STAT proteins are directly phosphorylated by JAK2 and control transcription of a variety of genes (Herrington *et al.* 2000, Zhu *et al.* 2001). GH has been reported to activate STAT1, STAT3, and mainly, STAT5, which regulates the transcription of the insulin-like growth factor-I (IGF-I) gene (Frank 2001, Zhu *et al.* 2001, Woelfle & Rotwein 2004). JAK2 also phosphorylates different adaptor proteins that lead to the activation of the mitogen-activated protein kinase (MAPK) ERK1/2 and phosphatidylinositol 3'-kinase (PI-3K)/Akt pathways (Zhu *et al.* 2001, Lanning & Carter-Su 2007). Moreover, GH has been shown to activate several signaling molecules, including epidermal growth factor receptor (EGFR), focal adhesion kinase (FAK), Src family members, Ras-like GTPases, p38 and JNK/SAPK MAP kinases, the mammalian target of rapamycin (mTOR), among others

(Zhu *et al.* 2001, Hayashi & Proud 2007, Lanning & Carter-Su 2007). Most downstream-signaling pathways activated by GH are dependent on JAK2 activity. However, JAK2-independent pathways have also been identified (Brooks *et al.* 2007). GH stimulation of NIH-3T3 cells resulted in the activation of the tyrosine kinase c-Src independently of the activity of JAK2, leading to the formation of GTP-bound RalA and RalB, which regulate the activation of ERK1/2 via phospholipase D (Zhu *et al.* 2002). In human leukemia cells, GH-activated Src, which then phosphorylated GHR and STAT5, independently of JAK2 activity (Manabe *et al.* 2006).

We have previously reported that the GH-induced JAK2/STAT5-signaling pathway is desensitized in the liver of transgenic mice overexpressing GH (González *et al.* 2002, Miquet *et al.* 2004, 2005). This is probably due, at least in part, to the overexpression of the cytokine-inducible SH2 domain containing protein (CIS), a member of the family of suppressors of cytokine-signaling (SOCS) proteins, which is involved in targeting receptor complexes for internalization and signal termination and was also reported to compete with STAT5b for binding sites in GHR (Ram & Waxman 2000, Landsman & Waxman 2005, Uyttendaele *et al.* 2007).

However, these animals exhibit phenotypic characteristics that indicate GH is indeed acting in the liver. For instance, liver GHR expression is increased, correlating with high serum bovine GH levels, and in accordance with the known ability of GH to upregulate its own receptor (McGrane *et al.* 1990, Aguilar *et al.* 1992, González *et al.* 2001, 2007, Iida *et al.* 2004). Moreover, circulating levels of IGF-I, which are primarily regulated by GH action in the liver, are also increased in GH-transgenic mice (Mathews *et al.* 1988, McGrane *et al.* 1990, Iida *et al.* 2004). Absolute and relative liver weight is higher in GH-transgenic than in control mice, accompanied by pathological alterations in the liver as a consequence of GH excess (Orian *et al.* 1989, Quaife *et al.* 1989, Hoefflich *et al.* 2001, Snibson 2002, Bartke 2003). Therefore, the objective of this study was to evaluate GH-signaling pathways that could be activated in the liver of GH-transgenic mice. These findings could lead to identify potential mechanisms leading to disproportional liver enlargement and other manifestations of GH action in the liver of these transgenic mice in spite of desensitization of a key pathway of GH signaling.

In contrast to the previous results for STAT5, STAT3 was constitutively phosphorylated in transgenic mice, although GH acute stimulation did not increase STAT3 phosphorylation level in these animals. An important finding in the present study is the overexpression of c-Src in transgenic mice, since this kinase could act as an alternative mediator to initiate signaling pathways independent of JAK2 activity. Other signaling mediators such as FAK, EGFR, Erk1/2, Akt, and mTOR also displayed elevated protein and basal phosphorylation levels. Thus, GH-overexpressing transgenic mice exhibit hepatic upregulation of signaling mediators implicated in the control of cell proliferation, survival, and motility.

Materials and Methods

Animals

Two models of GH overexpression were used. All the experiments were performed in the phosphoenolpyruvate carboxykinase (PEPCK)-bGH transgenic mouse model, and the results were later confirmed in the Mt-hGHRH transgenic mouse model.

PEPCK-bGH mice containing the bovine GH (bGH) gene fused to control sequences of the rat PEPCK gene (McGrane *et al.* 1990) were derived from animals kindly provided by Drs T E Wagner and J S Yun (Ohio University, Athens, OH, USA). The hemizygous transgenic mice were derived from a founder male and were produced by mating transgenic males with normal C57BL/6 × C3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Matings produced approximately equal proportion of transgenic and normal progeny. Normal siblings of transgenic mice were used as controls. Transgenic Mt-hGHRH animals were derived from animals originally produced by Mayo *et al.* (1988) and kindly provided by Dr J Hyde. Adult transgenic mice and their

normal siblings were produced by mating hemizygous male carriers of the human GH-releasing hormone (hGHRH) gene under the control of the metallothionein (Mt) promoter with normal C57BL/6J × C3H/J F1 females.

Transgenic animals had markedly accelerated postweaning growth, leading to a significant increase in body weight. Female adult animals (4–6 months old) were used. The mice were housed 3–5 per cage in a room with controlled light (12-h light/day) and temperature ($22 \pm 2^\circ\text{C}$). The animals had free access to food (Lab Diet Formula 5001; PMI 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 with institutional regulations.

Reagents

Highly purified ovine GH (oGH) from pituitary origin was obtained through the National Hormone and Pituitary Program, NIDDK, NIH (Bethesda, MD, USA). BSA-fraction V and protein G-Sepharose were obtained from Sigma Chemical Co. and polyvinylidene difluoride (PVDF) membranes and ECL-Plus from Amersham Biosciences. Secondary antibodies conjugated with horseradish peroxidase and antibodies anti-FAK (A-17) and anti-STAT5 (C-17) were purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA). Antibodies anti-phospho-STAT5a/b Tyr694/696 and anti-phospho-FAK Tyr397 were from Upstate Laboratories (Lake Placid, NY, USA). Antibodies anti-phospho-Akt Ser473, anti-Akt, anti-p44/42 MAP kinase, anti-phospho-p44/42 MAP kinase Thr202/Tyr204, anti-phospho-FAK Tyr925, anti-phospho-STAT3 Tyr705, anti-phospho-Src Tyr416, anti-nonphospho-Src Tyr416, anti-phospho-Src Tyr527, anti-nonphospho-Src Tyr527, anti-EGFR, anti-phospho-EGFR Tyr845, anti-phospho-mTOR Ser2448, and anti-mTOR were from Cell Signaling Technology Inc. Antibody anti-STAT3 was purchased from Transduction Laboratories (Lexington, KY, USA) and mouse monoclonal antibody (mAb) 327 against c-Src was kindly provided by Dr J Martín-Pérez (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain). All other chemicals were of reagent grade.

Serum GH determination

Mouse and bovine GHs were determined by RIA as described previously (González *et al.* 2007, Sotelo *et al.* 2008) using RIA kits obtained through the National Hormone and Pituitary Program, NIDDK, NIH.

Preparation of liver extracts and immunoprecipitation

The mice were fasted overnight and injected i.p. with 5 mg oGH per kg of body weight in 0.2 ml of 0.9% NaCl. Normal and transgenic mice were injected with saline to evaluate basal conditions. Mice were killed by cervical dislocation under isoflurane anesthesia 7.5 min after GH injection. The livers

were removed and homogenized in a ratio of 0.1 g/1 ml in buffer composed of 1% Triton, 100 mM HEPES, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.035 trypsin inhibitory units/ml aprotinin (pH 7.4) at 4 °C. Liver homogenates were centrifuged at 100 000 *g* for 40 min at 4 °C to remove insoluble material. Protein concentration of supernatants was determined by the method of Bradford (1976). An aliquot of solubilized liver was diluted in Laemmli buffer, boiled for 5 min, and stored at -20 °C until electrophoresis.

For immunoprecipitation, 4 mg solubilized liver protein were incubated at 4 °C overnight with 4 µl anti-c-Src mAb 327 antibody in a final volume of 0.4 ml. Additional samples were incubated in the absence of immunoprecipitating antibody in order to corroborate that the proteins precipitated were specifically recognized by the antibody and not by protein G-Sepharose. After incubation, 25 µl protein G-Sepharose (50%, v/v) were added to the mixture. The preparation was further incubated with constant rocking for 2 h at 4 °C and then centrifuged at 3000 *g* for 1 min at 4 °C. The supernatant was discarded and the precipitate was washed three times with buffer containing 50 mM Tris, 10 mM sodium vanadate, and 1% Triton X-100 (pH 7.4). The final pellet was resuspended in 50 µl Laemmli buffer, boiled for 5 min, and stored at -20 °C until electrophoresis.

In vitro Src tyrosine kinase assay

Src kinase activity was measured using an *in vitro* kinase assay kit from Upstate Biotechnology that is designed to measure the phosphotransferase activity of Src kinase in immunoprecipitates and column fractions. The assay was performed according to the manufacturer's instructions. Briefly, 2 mg protein in a final volume of 0.2 ml were immunoprecipitated from liver solubilizates with 2 µl anti-c-Src mAb 327 antibody. The immunoprecipitates were washed three times with 0.5 ml ice-cold buffer used for liver solubilization and three times with Tris-HCl 50 mM (pH 7.4). Beads were then resuspended in 10 µl kinase reaction buffer and 10 µl substrate peptide (150 µM final concentration). Subsequently, 10 µl of [γ -³²P]ATP stock were added and the reaction was incubated for 10 min at 30 °C with agitation. To precipitate the peptide, 20 µl of 40% tri-chloro acetic acid were added and the reaction was incubated for 5 min at room temperature. An aliquot of 25 µl was transferred to the center of a numbered P81 paper square, which was then washed three times with 0.75% phosphoric acid and once with acetone. The squares were transferred to a scintillation vial with 4 ml scintillation cocktail and the level of radioactivity was determined in a scintillation counter. A sample that contains no enzyme (i.e. no immunoprecipitating antibody) was used as a background control, and a sample that contains no substrate peptide was also included as a control. The values obtained for both controls were similar. The activity of each sample was

corrected by the activity of the control sample with no enzyme (background control).

Western blot analysis

Samples were subjected to electrophoresis in SDS-polyacrylamide gels using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Electrotransference of proteins from gel to nitrocellulose membranes was performed for 1 h at 100 V (constant) using the Bio-Rad miniature transfer apparatus in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol (pH 8.3). To reduce non-specific antibody binding, membranes were incubated 2 h at room temperature in Tween-Tris buffered saline (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. 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 with T-TBS. Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL-Plus, Amersham Biosciences) and images were scanned with STORM 860 (Amersham, Biosciences). Band intensities were quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Silver Spring, MD, USA). Additional membranes were analyzed by chemiluminescence prior to incubation with the primary antibody, to determine that the reactive band observed in the immunoblotting corresponds to a protein recognized specifically by the primary antibody (data not shown).

After the phosphorylation status of a protein was determined, the same membrane was then reprobed to assess the protein level. Membranes were washed 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 while shaking, washed with deionized water, and blocked with BSA.

Real-time reverse transcriptase PCR

Total hepatic RNA was extracted using the phenol chloroform method (Chomczynski & Saachi 1987). cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad) and the relative expression of the genes was analyzed by real-time PCR as described previously (Masternak *et al.* 2005). Table 1 shows the sequence of the primers used. β 2-microglobulin was used as a housekeeping gene and the relative expression levels were calculated according to the formula $2^{A-B}/2^{C-D}$ (A = cycle threshold (C_t) number of the gene of interest in the first control sample, B = C_t number of the gene of interest in each sample, C = C_t number of the housekeeping gene in the first control sample, D = C_t number of the housekeeping gene in each sample), as described previously (Masternak *et al.* 2005, Wang *et al.* 2007). The relative expression of the first normal sample was expressed as 1 and the relative expression of all other samples was calculated using this equation. The results from the

Table 1 Sequence of the primers used in real-time reverse transcriptase PCR

	GenBank accession no.	Sense primers (5′–3′)	Antisense primers (5′–3′)	Predicted PCR product size
Gene				
c-Src	NM_009271	GAACCTATAGGGACTGTGTG	TGAAGCCCTTCCATGCTCCA	139
EGFR	NM_207655	CGGATATGGACTTACAGAGCCATC	TGGCAGTTCTCCTCTCCTCCT	97
FAK	NM_007982	CGTGTGGATGTTTGGTGTGTGT	TCCCCCATTTTCAATTCGACCG	106
Akt1	NM_009652	TACAACCAGGACCACGACAA	TGATCTCCTTGGCATCCTCA	160
mTOR	NM_020009	CTTCGTGCCTGTCTGATTCT	CAAGGCTCCGTGGATTTCGAT	158
Erk2	NM_011949	CAGCACCTACGAATGACCA	TGATTGGAAGGCTTGAGGTCACG	110
β2-M	NM_009735	AAGTATACTACGCCACCCA	AAGACCAGTCCTTGCTGAAG	162

β2-M, β2-microglobulin.

normal group were averaged and all the results were then divided by this average to get the fold change of expression of each gene compared with normal mice group.

Statistical analysis

Experiments were performed analyzing all the groups of animals in parallel, *n* representing the number of different individuals used in each group. Results are presented as mean ± s.e.m. 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). Student’s *t*-test was used when the values of two groups were analyzed. Data were considered significantly different if *P* < 0.05.

Results

Animal characteristics

Serum GH concentration along with body and liver weight values for normal and transgenic mice are shown in Table 2 and are consistent with previous reports (Miquet *et al.* 2004, González *et al.* 2007, Sotelo *et al.* 2008). The elevation in GH levels is accompanied by an increment in both body and liver weight in transgenic mice. However, the relative increase in liver weight (3.1 fold) is higher than the one observed for body

weight (1.76-fold), reflecting that transgenic mice exhibit hepatomegaly.

STAT5 and STAT3 phosphorylation and protein content

GH treatment induced STAT5a/b phosphorylation at tyrosine 694/699 in normal mice, but not in PEPCK-bGH transgenic animals, in accordance with our previous reports (Miquet *et al.* 2004, 2005). STAT5 protein content was similar in all groups, with no differences in the basal phosphorylation levels of this protein between normal and transgenic mice (Fig. 1A–C). Although STAT5 is the predominant STAT utilized by GH, we have previously described that it is desensitized in the liver of GH-overexpressing transgenic mice liver (González *et al.* 2002, Miquet *et al.* 2004, 2005). Thus, it was of interest to determine whether STAT3 activation was also impaired in transgenic mice liver.

To assess STAT3 activation status, solubilized livers were subjected to western blotting analyses with an antibody that recognizes STAT3 when phosphorylated at Tyr705, a modification that activates it. Normal mice stimulated with GH displayed a marked increase in STAT3 phosphorylation at this site, while transgenic mice did not show this response to hormone treatment (Fig. 1D). Basal phosphorylation levels seemed slightly higher in GH-transgenic than in normal animals (Fig. 1D), while STAT3 protein content was similar in normal and transgenic mice and did not change with GH treatment (Fig. 1E). In an attempt to better discriminate the basal phosphorylation levels of this protein, only samples from

Table 2 Growth hormone (GH) circulating concentration, body weight, and liver weight in transgenic and normal siblings mice. GH serum levels were determined by specific RIA for mouse. Values are mean ± s.e.m. (number of animals per group)

Mice models	PEPCK-bGH		Mt-GHRH	
Genotype	Normal	Transgenic	Normal	Transgenic
GH serum levels (ng/ml)	4 ± 1 (6) ^a	1364 ± 100 (9) ^{b,*}	6 ± 2 (7) ^a	1994 ± 582 (8) ^{a,*}
Body weight (g)	25 ± 1 (10)	44 ± 2 (10)*	28 ± 1 (7)	47 ± 2 (8)*
Liver weight (g)	1.0 ± 0.1 (10)	3.1 ± 0.2 (10)*	ND	ND

GH^a or bovine GH^b. **P* < 0.001 versus normal mice. ND, not determined.

Figure 1 Normal (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)), killed after 7.5 min and the livers were removed. Equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to immunoblot analysis. Representative results of immunoblots with (A and C) anti-phospho-STAT5 Tyr694/696, (B) anti-STAT5, (D and F) anti-phospho-STAT3 Tyr705, or (E) anti-STAT3 are shown. The blots shown in (A and B) or (D and E) correspond to the reblotting of the same membranes used for phospho-STAT antibody with an antibody specific for the protein in analyses. Quantification was performed by scanning densitometry and expressed as % of values measured in (A, D) GH-stimulated normal mice or (B, C, E, F) non-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (n) of different individuals, run in separate experiments. Different letters denote significant difference at $P < 0.05$. MWM, molecular weight markers.

animals that were not hormone treated were run in parallel (Fig. 1F). Transgenic mice displayed significantly higher STAT3 basal phosphorylation levels than normal controls (approx. fourfold) suggesting that even when this signaling pathway does not respond to acute GH stimuli, its basal activation is higher in GH-overexpressing transgenic mice. The pattern of phospho-STAT3 Tyr705 immunoreactive bands seems to be different between normal stimulated mice, which evidence a clear doublet, and transgenic animals, which only show an increase in the intensity of the band that would correspond to the lower band of the doublet. The upper band of the doublet – only observed for the normal stimulated group – may reflect a delayed electrophoretic motility, possibly due to phosphorylation at additional sites (Ram *et al.* 1996). Therefore, GH acute stimulation in normal mice and prolonged exposure to high GH levels in transgenic mice may induce different phosphorylation status of STAT3. It is noteworthy that STAT3 antibody is immunoreactive to one ~ 91 kDa protein corresponding to the lower band of the doublet. Therefore, the upper band of the doublet is either

non-specific or anti-STAT3 antibody does not recognize this modified form of STAT3.

c-Src phosphorylation, protein content, and kinase activity

The control of the phosphorylation status of c-Src, exerted by a balance between phosphorylation and dephosphorylation of positive and negative regulatory residues, is an important mechanism of regulation of its kinase activity. c-Src phosphorylation was assayed by immunoprecipitation of solubilized livers with a specific antibody against c-Src followed by western blotting with antibodies that recognize Src family members either when phosphorylated at tyrosine 416 or 527, or that only detect the kinase when those residues are not phosphorylated. Src phosphorylation at Tyr416, a positive regulatory autophosphorylation site (Bjorge *et al.* 2000, Boggon & Eck 2004), was markedly increased in PEPCK-bGH transgenic mice compared with their normal controls. Acute GH treatment of normal mice seemed to moderately increase the phosphorylation of Src at this residue,

although it did not reach statistical significance; for transgenic animals GH treatment did not change the phosphorylation at this site (Fig. 2A). When Src that was not phosphorylated at this site was analyzed, transgenic mice exhibited higher levels with respect to their normal siblings; GH treatment did not produce any change in either group (Fig. 2D). Src phosphorylation at Tyr527 constitutes a negative regulatory mechanism of the kinase activity (Bjorge *et al.* 2000, Boggon & Eck 2004). A similar pattern to that of the activating residue was observed for Tyr527; that is, both the levels of phosphorylation of this residue and the levels of Src not phosphorylated at this specific site were notably increased in transgenic mice, with no variations upon acute GH treatment (Fig. 2B and E).

The protein content of c-Src, analyzed by immunoprecipitation and western blotting with the same specific antibody, was higher in transgenic mice (~3.7-fold; Fig. 2C). In addition, the tyrosine kinase activity of c-Src was determined by an *in vitro* kinase assay kit after immunoprecipitation of c-Src. Transgenic mice presented higher kinase activity than

normal mice (approx. fourfold), reflecting the increase in c-Src protein levels (Fig. 2F).

These results indicate that c-Src is upregulated in transgenic mice liver, and that the observed increases in the kinase activity and the phosphorylation status are mainly a consequence of the higher c-Src protein content in transgenic mice.

FAK phosphorylation and protein content

The phosphorylation and the protein content of FAK were assayed by western blotting of solubilized livers with specific antibodies. Tyr397 is a site that is autophosphorylated upon FAK activation and serves as a binding site for Src family kinases (Parsons 2003). PEPCK-bGH transgenic mice showed twofold higher basal levels of FAK phosphorylated at Tyr397 while GH treatment did not produce any change. For normal mice, a moderate increase was observed upon GH treatment, but this was not statistically significant (Fig. 3A). Src family members, once recruited to FAK, may phosphorylate it at

Figure 2 Normal (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)), killed after 7.5 min and the livers were removed. Equal amounts of solubilized liver protein were immunoprecipitated with anti-c-Src antibody, separated by SDS-PAGE and subjected to immunoblot analysis using (A) anti-phospho-Src Tyr416, (B) anti-phospho-Src Tyr527, (C) anti-c-Src, (D) anti-nonphospho-Src Tyr416 or (E) anti-nonphospho-Src Tyr527. (C) In the blot, a negative control (c –) is shown, which corresponds to a sample from transgenic mice in which immunoprecipitating antibody was not added. Quantification was performed by scanning densitometry and expressed as % of values measured in non-stimulated transgenic mice. (F) In addition, c-Src immunoprecipitated complexes were washed and subjected to an *in vitro* kinase assay. Data are the mean $\pm$ s.e.m. of the indicated number of subsets (*n*) of different individuals, run in separate experiments (three to four independent experiments for western blots, two independent experiments for the kinase activity assay). Different letters denote significant difference at $P < 0.05$. Representative immunoblots are also shown. MWM, molecular weight markers.

Figure 3 Normal mice (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)) and after 7.5 min and the livers were excised. Extracts were prepared and equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to immunoblot analysis with (A) anti-phospho-FAK Tyr397, (B) anti-phospho-FAK Tyr925, or (C) anti-FAK antibodies. Quantification was performed by scanning densitometry and expressed as % of values measured in non-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (*n*) of different individuals run in three to four separate experiments. Different letters denote significant difference at $P < 0.05$. Representative immunoblots are also shown. MWM, molecular weight markers.

different residues, such as Tyr925, thus promoting further activation. The basal phosphorylation of this site was also twofold higher in transgenic mice; GH stimulation produced a 50% increase in normal mice, while no changes were detected in transgenic mice (Fig. 3B). FAK protein levels were also increased in transgenic mice when compared with their normal littermates (Fig. 3C), indicating that FAK is upregulated in transgenic mice liver.

EGFR protein content

EGFR protein content and phosphorylation at Tyr845 were analyzed by western blotting of liver solubilizates with specific antibodies. c-Src was reported to interact with EGFR and to phosphorylate it at Tyr845, stabilizing the enzyme in the activated state (Biscardi *et al.* 2000). EGFR protein levels were higher in PEPCK-bGH transgenic than in normal mice (Fig. 4B). Phosphorylation at Tyr845 was also increased in transgenic mice, but no phosphorylation was detected upon acute GH stimulation in normal or transgenic mice (Fig. 4A).

Akt and mTOR phosphorylation and protein content

The phosphorylation and the protein content of Akt were analyzed by western blotting of solubilized livers. Akt phosphorylation at Ser473, an activating residue, was increased approximately twofold in PEPCK-bGH transgenic mice compared with normal controls. The phosphorylation at this site did not significantly vary with acute GH stimulation either in normal or transgenic mice, although a slight but statistically non-significant increase could be observed in normal animals (Fig. 5A). Akt protein content was also twofold higher in transgenic mice (Fig. 5B), indicating that this kinase is also upregulated in transgenic mice liver.

GH was reported to activate rapid protein synthesis via mTOR signaling (Hayashi & Proud 2007). The phosphorylation and protein content of mTOR were analyzed by western blotting of liver solubilizates. Phosphorylation at Ser2448, which is catalyzed by the PI-3K/Akt pathway, was twofold higher in PEPCK-bGH transgenic than in normal mice (Fig. 5C). Similarly, mTOR protein content was increased in transgenic mice compared with their normal controls (Fig. 5D).

Erk phosphorylation and protein content

Erk1 and Erk2 (44 and 42 kDa respectively) are activated by phosphorylation at Thr202 and Tyr204. Western blotting of solubilized livers with an antibody that specifically recognizes the enzymes phosphorylated in these residues revealed that Erk2 phosphorylation was increased in PEPCK-bGH transgenic mice, while Erk1 presented no variations between normal and transgenic animals. GH stimulation did not change Erk1/2 phosphorylation, either in normal or transgenic mice (Fig. 6A).

Erk1 and Erk2 protein content were both increased in transgenic mice (Fig. 6B). Erk2-increased phosphorylation corresponds in magnitude with the higher levels of this protein observed in transgenic mice (~ 1.5 -fold). However, Erk1 protein upregulation was not accompanied with a parallel increment of its phosphorylation.

Signaling mediators in the Mt-hGHRH transgenic mouse model

In order to confirm the aforementioned results in another GH overexpression mouse model, the signaling mediators determined in PEPCK-bGH transgenic mice were also assayed in the Mt-hGHRH transgenic mouse model. As observed for PEPCK-bGH transgenic mice, GH acute

Figure 4 Normal mice (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)) and after 7.5 min the livers were excised. Extracts were prepared and equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to (A) immunoblot analysis with anti-phospho-EGFR Tyr845 and (B) reprobed with anti-EGFR antibodies. Quantification was performed by scanning densitometry and expressed as % of values measured in non-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (*n*) of different individuals run in four separate experiments. Different letters denote significant difference at $P < 0.05$. Representative results of immunoblots are shown. MWM, molecular weight markers.

Figure 5 Normal (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)). After 7.5 min, the livers were excised and extracts were prepared. Equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to immunoblot analysis with (A) anti-phospho-Akt Ser473, (B) anti-Akt, (C) anti-phospho-mTOR Ser2448, or (D) anti-mTOR antibodies. Blots shown in (A and B) or (C and D) correspond to the same membrane incubated with the anti-phospho-specific antibody and reprobed with an antibody against the protein of interest. Quantification was performed by scanning densitometry and expressed as % of the corresponding values in non-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets of different individuals (*n*) run in four separate experiments. Different letters denote significant difference at $P < 0.05$. Representative immunoblots are also shown. MWM, molecular weight markers.

stimulation did not significantly increase Tyr705 phosphorylation of STAT3 in Mt-hGHRH transgenic mice but induced a marked response in normal littermates (Fig. 7A); no variations in STAT3 protein content were observed between normal and transgenic animals (Fig. 7B). However, when only basal STAT3 phosphorylation levels were analyzed, significantly higher phosphorylation at Tyr705 could be detected in transgenic mice compared with normal animals (Fig. 7C). In accordance with the results obtained for PEPCK-bGH transgenic mice, Src, FAK, Akt, Erk1/2, EGFR, and mTOR protein contents were also increased in Mt-hGHRH transgenic mice liver (Fig. 7D–I). Acute GH stimulation did not change the level of any of these proteins, in accordance with the results obtained with PEPCK-bGH transgenic mice (data not shown).

Real-time reverse transcriptase PCR

In order to evaluate if the observed protein overexpression of the signaling mediators was related to increased mRNA expression, real-time RT-PCR assay was performed in the livers from the PEPCK-bGH transgenic mouse model. Table 1 shows the primers used and Table 3 the relative expression of the genes analyzed. Only c-Src mRNA levels were significantly higher in transgenic mice liver (approx. tenfold). EGFR expression was 60% increased in transgenic mice, but it did not reach statistical significance. FAK and Erk2 presented a similar expression in normal and transgenic animals, while Akt1 and mTOR mRNA levels were significantly lower in transgenic mice. For Akt, only the isoform Akt1 was determined because it is the predominant isoform in the most tissues. For Erk, we only determined Erk2 in order to simplify the experiments, as both Erk1 and Erk2 proteins were upregulated.

Discussion

Transgenic mice overexpressing GH show increased body weight, organomegaly – particularly in the liver – increased circulating IGF-I and hepatic IGF-I mRNA levels, among other alterations (Table 2; Mathews *et al.* 1988, McGrane *et al.* 1990, Sotelo *et al.* 1998, Hoefflich *et al.* 2001, Bartke 2003, Iida *et al.* 2004). The disproportional increase in liver weight is due to hypertrophy and hyperplasia (Orian *et al.* 1989, Hoefflich *et al.* 2001, Snibson 2002, Bartke 2003), most likely as a consequence of a direct action of GH on hepatocytes rather than effects mediated by IGF-I (Quaife *et al.* 1989, Bartke 2003).

PEPCK-bGH transgenic mice used in this work exhibit lifelong elevated bGH levels with a consequent increase in body weight (Sotelo *et al.* 1995, 1998, Miquet *et al.* 2004). In accordance with the positive regulation that chronic GH increase exerts over its receptor (McGrane *et al.* 1990, Iida *et al.* 2004, González *et al.* 2007), hepatic levels of GH receptor are increased in transgenic mice overexpressing GH (Aguilar *et al.*

Figure 6 Normal (N) and PEPCK-bGH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)). After 7.5 min, the livers were excised and extracts were prepared. Equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to immunoblot analysis with (A) anti-phospho-Erk1/2 Thr202/Tyr204 or (B) anti-Erk1/2 antibodies. Quantification was performed by scanning densitometry and expressed as % of the corresponding values in non-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated number of subsets of different individuals (*n*) run in four separate experiments. Different letters denote significant difference at $P < 0.05$. Representative immunoblots are shown.

1992, González *et al.* 2001, Miquet *et al.* 2004). In previous works, we have already described that the JAK2/STAT5-signaling pathway is desensitized in the liver of GH-overexpressing mice (González *et al.* 2002, Miquet *et al.* 2004, 2005). The aim of the present work was to evaluate other signaling mediators in the liver of transgenic mice to assess whether the desensitization is extended to other GH-signaling pathways and to detect possible alternative pathways that may be activated in transgenic mice liver, which could account for the hepatic alterations observed in GH-transgenic mice.

Signal transducers and activators of transcription participate in diverse cell processes, such as differentiation, proliferation, and apoptosis (Calò *et al.* 2003). GH activates STAT1, STAT3, STAT5a, and STAT5b through tyrosine phosphorylation by JAK2 (Zhu *et al.* 2001, Lanning & Carter-Su 2007). Recently, it was reported that c-Src could phosphorylate STAT5 in response to GH independently of JAK2 activity in human leukemia cells (Manabe *et al.* 2006). Src kinases may directly phosphorylate STAT1, STAT3, and STAT5 (Zhu *et al.* 2001, Silva 2004). Although STAT3 did not respond to a massive, acute GH stimulus in GH-transgenic mice, its basal activation was higher in transgenic than in normal mice liver. This result is different from that of STAT5, as no differences could be detected in the phosphorylation levels between normal and transgenic mice, reflecting that STAT5 activation is not basally increased in GH-overexpressing transgenic mice. The lack of response to an acute exogenous GH stimulus found for transgenic mice is not surprising since these animals already exhibit high circulating GH levels, which may render these animals less sensitive to the exogenous administered. At high concentrations, GH action may decrease because the

formation of complexes with stoichiometry GH₁:GHR₁ is favored, thus inhibiting the formation of the active complex GH₁:GHR₂ and the consequent activation of the signaling pathways (Fuh *et al.* 1992, Frank 2002). It is also probable that the chronic persistency of high GH levels – opposed to the physiological pulsatile pattern – may be related to the observed effects. In fact, we have previously reported that CIS, a member of the family of SOCS proteins which is induced by GH and negatively regulates its signaling, is upregulated in GH-transgenic mice liver (González *et al.* 2002, Miquet *et al.* 2004). However, it should be noted that the employed GH dose (5 µg/g body weight) is high even for GH-transgenic mice. At this high GH dose, the formation of complexes with stoichiometry GH₁:GHR₁ probably occurs, but nevertheless the activation of STAT5 and STAT3 were perfectly detected in normal mice, indicating that GHR dimerization also occurs at these high concentrations, likely due to concomitant increase in GHRs.

An important finding of this work is that c-Src mRNA and protein are both upregulated in the liver of GH-overexpressing transgenic mice, as this kinase may initiate signaling cascades independent of JAK2 (Zhu *et al.* 2002, Manabe *et al.* 2006, Brooks *et al.* 2007). Members of the Src family kinases are non-receptor tyrosine kinases involved in the signaling of many cellular processes, including cell growth, proliferation, differentiation, motility, adhesion, and survival (Thomas & Brugge 1997, Parsons & Parsons 2004). All family members contain a C-terminal tail bearing an autoinhibitory phosphorylation site, as well as a positive regulatory autophosphorylation site that is present in the activation loop. Src activity can also be regulated through its interaction with

Figure 7 Normal mice (N) and Mt-hGHRH transgenic (T) mice were injected i.p. with normal saline (non-stimulated (–)) or oGH (5 mg/kg) (GH-stimulated (+)) and after 7.5 min, the livers were excised. Extracts were prepared and equal amounts of solubilized liver protein were separated by SDS-PAGE and subjected to immunoblot analysis with (A and C) anti-phospho-STAT3 Tyr705, (B) anti-STAT3, (D) anti-Src, (E) anti-FAK, (F) anti-EGFR, (G) anti-Akt, (H) anti-mTOR, and (I) anti-Erk1/2 (I) antibodies. Quantification was performed by scanning densitometry and expressed as % of values measured in GH-stimulated normal mice (A) or as % of normal mice values (B–I). In (I), each column represents the sum of Erk1 and Erk2 values, as the pattern observed for both proteins was similar. Data are the mean $\pm$ S.E.M. of the indicated number of subsets (n) of different individuals run in two separate experiments. Different letters denote significant difference at $P < 0.05$. Representative immunoblots are shown.

various ligands that bind to the SH2 or SH3 domains of Src, thus disturbing the intramolecular interactions that maintain Src in the inactive conformation (Bjorge *et al.* 2000, Boggon & Eck 2004). Src kinase activity, analyzed by an *in vitro* kinase assay, was increased to a similar extent than the protein content of c-Src determined by western blotting. Moreover, the phosphorylation of the positive and the negative regulatory sites was increased in transgenic mice in a similar magnitude.

These results suggest that the observed increase in c-Src kinase activity in transgenic mice is mainly due to the overexpression of this protein and not to an altered balance between phosphorylation of regulatory residues. Elevated Src activity due only to an increase in c-Src protein levels with no alterations of the specific activity of the enzyme was found in human breast neoplasias (Biscardi *et al.* 2000). Although it has been reported that GH was able to induce c-Src activation in

Table 3 Real-time reverse transcriptase PCR in phosphoenolpyruvate carboxykinase-bovine growth hormone (PEPCK-bGH) transgenic mice. Values are means $\pm$ S.E.M. ($n=7$)

Genotype	Normal	Transgenic
c-Src	1.0 $\pm$ 0.4	9.6 $\pm$ 2.8*
EGFR	1.0 $\pm$ 0.1	1.6 $\pm$ 0.5
FAK	1.0 $\pm$ 0.1	1.0 $\pm$ 0.3
Akt1	1.0 $\pm$ 0.2	0.3 $\pm$ 0.1*
mTOR	1.0 $\pm$ 0.2	0.5 $\pm$ 0.2*
Erk2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2

* $P<0.05$ versus normal mice.

different cell lines (Zhu *et al.* 2001, 2002, Manabe *et al.* 2006, Lanning & Carter-Su 2007), no differences in the phosphorylation status or kinase activity were observed upon GH stimulation in the liver, either in normal or transgenic animals.

FAK is implicated in cell adhesion, migration, growth, and survival (Parsons 2003). This kinase is activated by integrin-mediated cell adhesion, leading to the autophosphorylation of Tyr397; this modification acts as a binding site for Src, resulting in Src activation, which further facilitates FAK maximal activation by phosphorylating it at Tyr925 (Abram & Courtneidge 2000, Parsons 2003, Schlaepfer & Mitra 2004). The activated signaling complex Src-FAK can be then implicated in the initiation of diverse signaling cascades, such as PI-3K, MAPK, and phospholipase (PLC)- γ pathways (Parsons 2003, Schlaepfer & Mitra 2004). FAK can also be activated by nonintegrin stimuli, including GH, which has been implicated in the reorganization of the actin cytoskeleton (Zhu *et al.* 2001, Lanning & Carter-Su 2007). Normal mice showed a moderate increase in the phosphorylation of both sites upon GH treatment, though it was only statistically significant for Tyr925 – the target of c-Src. FAK signaling was constitutively upregulated in transgenic mice liver, which exhibited higher FAK protein hepatic levels, accompanied by a concomitant increment in the phosphorylation of both residues. As FAK is implicated in several signaling cascades, it could then allow the propagation of GH signaling by diverse pathways (Zhu *et al.* 2001).

GH may signal via members of the EGFR subfamily of receptor tyrosine kinases, which regulate cell differentiation, proliferation, survival, and motility (Holbro *et al.* 2003). GH may stimulate Tyr phosphorylation of EGFR and its association with Grb2, with the consequent stimulation of MAPK activity, in the liver (Yamauchi *et al.* 1997, 1998). JAK2 has been reported to phosphorylate EGFR upon GH stimulation, although its catalytic activity was not increased (Zhu *et al.* 2001). c-Src may directly interact with EGFR, catalyzing Tyr845 phosphorylation, which would be involved in the regulation of EGFR-mediated mitogenesis and transformation (Biscardi *et al.* 1999). Transgenic mice exhibited an important increase in EGFR protein levels compared with normal controls, in accordance with previous reports indicating that GH upregulates the hepatic

concentration of EGFR in rodents (Jansson *et al.* 1988, Ekberg *et al.* 1989). No phosphorylation at Tyr845 could be detected upon GH stimulation in normal or transgenic mice, indicating that under the experimental conditions used, acute stimulation with GH did not induce the phosphorylation of this site. Nevertheless, chronic GH stimulation did elevate EGFR phosphorylation at Tyr845 in transgenic mice.

Both EGFR and the Src-FAK complex may signal via PI-3K/Akt pathway, which is central in many cellular responses, such as cell proliferation and survival, cellular metabolism, and cytoskeletal reorganization (Zhu *et al.* 2001, Holbro *et al.* 2003, Parsons 2003). GH has been shown to activate the PI-3K/Akt pathway by different mechanisms (Zhu *et al.* 2001, Lanning & Carter-Su 2007). GH stimulates the phosphorylation of insulin receptor substrates (IRS)-1, -2, and -3, which leads to the association with p85 subunit of PI-3K, activating it. This phosphorylation may be mediated directly by JAK2, but GH may utilize FAK and associated c-Src kinase as well to phosphorylate IRS (Zhu *et al.* 2001). Activation of PI-3K is related to the GH stimulation of glucose transport and to the activation of Akt, a serine/threonine kinase implicated in cellular proliferation, differentiation, metabolism, and survival (Nicholson & Anderson 2002, Song *et al.* 2005, Lanning & Carter-Su 2007). Transgenic mice displayed an increment both in Akt protein content and in the phosphorylation of the activating residue Ser473, indicating an upregulation of this pathway in transgenic animals. In normal mice, GH induced an apparent but statistically non-significant increase in Akt phosphorylation levels. Growth factors and hormones may activate mTOR by phosphorylation via the PI-3K/Akt-signaling pathway (Hidalgo & Rowinsky 2000, Dann *et al.* 2007). Recently, GH was reported to activate rapid protein synthesis via mTOR signaling (Hayashi & Proud 2007). Transgenic mice exhibited both increased protein levels and phosphorylation of mTOR, which would result in higher protein synthesis. Interestingly, PEPCK-bGH transgenic mice are insulin resistant, in accordance with the well-established fact that the elevation of circulating GH levels causes hyperinsulinemia and insulin resistance (Davidson 1987, Jorgensen *et al.* 2004, Dominici *et al.* 2005). The hepatic activity of PI-3K was dramatically increased in these transgenic mice, but insulin stimulation did not further increase it (Dominici *et al.* 1999). The PI-3K/Akt-signaling pathway appears to be a point of crosstalk between GH and insulin signaling, so the alterations observed at this level in PEPCK-bGH transgenic mice could be a result of the combination of the effects of the chronic elevation of both hormones these mice exhibit (Dominici *et al.* 2005). In any case, as the hyperinsulinemia is secondary to the overexpression of GH, the primary cause of these findings would be the prolonged exposure to high GH levels, which would either direct or indirectly produce these alterations. In addition to the hyperinsulinemia, transgenic mice exhibit altered levels of circulating IGF-1 and adipocytokines, such as adiponectin and resistin (Wang *et al.* 2007), which may also contribute in some point to the upregulation of the mediators analyzed.

MAP kinases stimulate DNA synthesis and promote cell-cycle progression and cell survival (Chang & Karin 2001, Chambard *et al.* 2007). GH may activate Erk1/2 (p44/42 MAPK) by different mechanisms, most of which involve JAK2 activity (Zhu *et al.* 2001). However, JAK2-independent mechanisms were reported by which GH may activate Erk1/2 in a Src-dependent way, via Ral and phospholipase D or via the c-Src-FAK-Grb2 complex (Zhu *et al.* 2001, 2002, Lanning & Carter-Su 2007). No variations in the phosphorylation of Erk1/2 were observed in normal mice upon stimulation with GH, suggesting that, at least for the experimental conditions used, GH does not significantly activate this pathway in the liver. Transgenic mice displayed a moderate elevation in Erk1/2 hepatic content, which was accompanied by a similar increment in the phosphorylation levels for p42, but p44 activation levels were similar to those of normal animals. These data suggest that hepatic Erk activity could be slightly elevated in GH-transgenic mice.

When the gene expression was analyzed by real-time RT-PCR, only c-Src displayed significantly elevated mRNA levels, suggesting that c-Src is transcriptionally regulated by GH, while in the case of mTOR and Akt1 mRNA levels were lower in transgenic mice. Therefore, the increased protein content of these signaling mediators may occur by post-transcriptional processes in each case. However, an elevation in the protein content would be physiologically important even if mRNA levels do not change.

In the PEPCK-bGH transgenic mouse model, a heterologous GH is overexpressed in non-pituitary tissues, including the liver. It could be speculated that the expression of large amounts of bGH in the liver could be involved in the alterations observed in this tissue. However, it was suggested that the intracellular overexpression of GH *per se* would not be responsible for the morphological alterations of hepatocytes characteristic of the liver pathology of GH-transgenic mice (Snibson 2002). To investigate if the results observed in PEPCK-bGH transgenic mice could be extended to other models of lifelong GH overexpression, the protein content of the signaling mediators evaluated was also assayed in Mt-hGHRH transgenic mice. These animals present a similar phenotype but exhibit chronically elevated GHRH levels, with a consequent increase in circulating endogenous GH from pituitary origin (Mayo *et al.* 1988, González *et al.* 2002, 2007). The constitutive phosphorylation of STAT3 and the upregulation of Src, FAK, EGFR, Akt, mTOR, and Erk1/2 were confirmed in Mt-hGHRH transgenic mice, indicating that the observed molecular alterations in the liver are probably a consequence of the chronically elevated GH levels these animals exhibit, and not a consequence of local GH production.

Studies performed in different lines of transgenic mice overexpressing GH revealed a disproportional increase in liver size due to hypertrophy and hyperplasia, with hepatocytes presenting morphological alterations such as large cell and nuclear size and intranuclear inclusions (Orian *et al.* 1989, Hoefflich *et al.* 2001, Bartke 2003). Transgenic mice that overexpress GH frequently develop liver tumors, mainly

hepatocellular carcinoma, most commonly observed in old animals (Orian *et al.* 1990, Snibson *et al.* 1999, Snibson 2002, Bartke 2003). Throughout lifespan, transgenic mice present high levels of hepatocellular replication, followed by the onset of hepatic inflammation, fibrosis, and cirrhosis, in many cases progressing to hepatocarcinoma (Orian *et al.* 1990, Snibson *et al.* 1999, Snibson 2002). The preneoplastic pathology in liver of GH-transgenic mice is similar to that present in humans at high risk of developing hepatic cancer (Snibson 2002, Thomas & Zhu 2005). A relationship between GH and cancer has been proposed, although further epidemiological investigation remains to be done (Jenkins & Besser 2001, Perry *et al.* 2006). Cancer cells show alterations in cytoskeletal organization, adhesion, motility, growth control, and survival. Many of the signaling pathways implicated in these events are upregulated in the liver of GH-overexpressing transgenic mice. These molecular alterations may thus be involved in the hypertrophy, hyperplasia, and morphological alterations observed in GH-overexpressing transgenic mice, frequently ending in malignant transformation at advanced ages (Orian *et al.* 1989, 1990, Snibson *et al.* 1999, Hoefflich *et al.* 2001, Snibson 2002, Bartke 2003). It has yet to be investigated if the observed upregulation of the signaling mediators occur in the same liver cell types or if some proteins are enriched in some cell populations. It would also be very interesting to study the expression of these proteins in the tumors that old transgenic mice develop.

The present findings indicate that GH-overexpressing transgenic mice exhibit upregulation in the liver of c-Src, FAK, EGFR, Akt, mTOR, and Erk1/2, and increased basal activation of STAT3. As these molecules are all signaling mediators of GH, the upregulation of these proteins may represent alternative pathways to JAK2/STAT5 that are constitutively activated in transgenic mice overexpressing GH, which may be implicated in the alterations observed in the liver of transgenic mice. Whether these observations are a direct effect of GH action or secondary to other endocrine or metabolic alterations transgenic mice exhibit remains to be determined, but it can be concluded from the study of two lines of GH-transgenic mice that sustained GH exposure to high GH levels is associated with exacerbated expression of several signaling mediators involved in proliferation, survival, and motility.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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References

- Abram CL & Courtneidge SA 2000 Src family tyrosine kinases and growth factor signaling. *Experimental Cell Research* **254** 1–13.
- Aguilar RC, Fernández HN, Dellacha JM, Calandra RS, Bartke A, Ghosh PK & Turyn D 1992 Somatotrophic and lactotropic receptors in transgenic mice expressing human and bovine growth hormone. *Transgenic Research* **1** 221–227.
- Bartke A 2003 Can growth hormone accelerate aging? Evidence from GH-transgenic mice. *Neuroendocrinology* **78** 210–216.
- Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH & Parsons SJ 1999 c-Src mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *Journal of Biological Chemistry* **274** 8335–8343.
- Biscardi JS, Ishizawa R, Silva CM & Parsons SJ 2000 Tyrosine kinase signaling in breast cancer – epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Research* **2** 203–210.
- Bjorge JD, Jakymiw A & Fujita DJ 2000 Selected glimpses into the activation and function of Src kinase. *Oncogene* **19** 5620–5635.
- Boggon TJ & Eck MJ 2004 Structure and regulation of Src family kinases. *Oncogene* **23** 7918–7927.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Brooks AJ, Wooh JW, Tunny KA & Waters MJ 2007 Growth hormone receptor; mechanism of action. *International Journal of Biochemistry and Cell Biology*. DOI:10.1016/j.biocel.2007.07.008.
- Calò V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N & Russo A 2003 STAT proteins: from normal control of cellular events to tumorigenesis. *Journal of Cellular Physiology* **197** 157–168.
- Chambard JC, Lefloch R, Pouyssegur J & Lenormand P 2007 Erk implication in cell cycle regulation. *Biochimica et Biophysica Acta* **1773** 1299–1310.
- Chang L & Karin M 2001 Mammalian MAP kinase signaling cascades. *Nature* **410** 37–40.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Dann SG, Selvaraj A & Thomas G 2007 mTOR Complex1–S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends in Molecular Medicine* **13** 252–259.
- Davidson MB 1987 Effects of growth hormone on carbohydrate and lipid metabolism. *Endocrine Reviews* **8** 115–131.
- Dominici FP, Cifone D, Bartke A & Turyn D 1999 Loss of sensitivity to insulin at early events of the insulin signaling pathway in the liver of growth hormone-transgenic mice. *Journal of Endocrinology* **161** 383–392.
- Dominici FP, Argentino DP, Muñoz MC, Miquet JG, Sotelo AI & Turyn D 2005 Influence of the crosstalk between growth hormone and insulin signaling on the modulation of insulin sensitivity. *Growth Hormone and IGF Research* **15** 324–336.
- Ekberg S, Carlsson L, Carlsson B, Billig H & Jansson JO 1989 Plasma growth hormone pattern regulates epidermal growth factor (EGF) receptor messenger ribonucleic acid levels and EGF binding in the rat liver. *Endocrinology* **125** 2158–2166.
- Frank SJ 2001 Growth hormone signaling and its regulation: preventing too much of a good thing. *Growth Hormone and IGF Research* **11** 201–212.
- Frank SJ 2002 Receptor dimerization in GH and Erythropoietin action: it takes two to tango, but how? *Endocrinology* **143** 2–10.
- Fuh G, Cunningham BC, Fukunaga R, Nagata S, Goeddel DV & Wells JA 1992 Rational design of potent antagonists to the human growth hormone receptor. *Science* **256** 1677–1680.
- González L, Sotelo AI, Bartke A & Turyn D 2001 Growth hormone (GH) and estradiol regulation of membrane-associated GH binding protein and GH receptors in GH releasing hormone transgenic mice. *Growth Hormone and IGF Research* **11** 34–40.
- González L, Miquet JG, Sotelo AI, Bartke A & Turyn D 2002 Cytokine inducible SH2 protein up-regulation is associated with desensitization of GH signaling in GHRH-transgenic mice. *Endocrinology* **143** 286–294.
- González L, Curto LM, Miquet JG, Bartke A, Turyn D & Sotelo AI 2007 Differential regulation of membrane associated-growth hormone binding protein (MA-GHBP) and growth hormone receptor (GHR) expression by growth hormone (GH) in mouse liver. *Growth Hormone and IGF Research* **17** 104–112.
- Hayashi AA & Proud CG 2007 The rapid activation of protein synthesis by growth hormone requires signaling through the mammalian target of rapamycin, mTOR. *American Journal of Physiology. Endocrinology and Metabolism* **292** E1647–E1655.
- Herrington J, Smit LS, Schwartz J & Carter-Su C 2000 The role of STAT proteins in growth hormone signaling. *Oncogene* **19** 2585–2597.
- Hidalgo M & Rowinsky EK 2000 The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* **19** 6680–6686.
- Hoeflich A, Nedbal S, Blum WF, Erhard M, Lahm H, Brem G, Kolb HJ, Wanke R & Wolf E 2001 Growth inhibition in giant transgenic mice by overexpression of insulin-like growth factor binding protein-2. *Endocrinology* **142** 1889–1898.
- Holbro T, Civenni G & Hynes NE 2003 The ErbB receptors and their role in cancer progression. *Experimental Cell Research* **284** 99–110.
- Iida K, Del Rincon JP, Kim DS, Itoh E, Nass R, Coschigano KT, Kopchick JJ & Thorner MO 2004 Tissue specific regulation of growth hormone (GH) receptor and insulin-like growth factor-I gene expression in the pituitary and liver of GH-deficient (*lit/lit*) mice and transgenic mice that overexpress bovine GH (bGH) or a bGH antagonist. *Endocrinology* **145** 1564–1570.
- Jansson JO, Ekberg S, Hoath SB, Beamer WG & Frohman LA 1988 Growth hormone enhances hepatic epidermal growth factor receptor concentration in mice. *Journal of Clinical Investigation* **82** 1871–1879.
- Jenkins PJ & Besser M 2001 Acromegaly and cancer: a problem. *Journal of Clinical Endocrinology and Metabolism* **86** 2935–2941.
- Jorgensen JO, Krag M, Jessen N, Norrelund H, Vestergaard ET, Møller N & Christensen JS 2004 Growth hormone and glucose metabolism. *Hormone Research* **62** 51–55.
- Landsman T & Waxman DJ 2005 Role of cytokine-induced SH2 domain-containing CIS in growth hormone receptor internalization. *Journal of Biological Chemistry* **280** 37471–37480.
- Lanning NJ & Carter-Su C 2007 Recent advances in growth hormone signaling. *Reviews in Endocrine and Metabolic Disorders* **7** 225–235.
- Manabe N, Kubota Y, Kitanaka A, Ohnishi H, Taminato T & Tanaka T 2006 Src transduces signaling via growth hormone (GH)-activated GH receptor (GHR) tyrosine-phosphorylating GHR and STAT5 in human leukemia cells. *Leukemia Research* **30** 1391–1398.
- Masternak MM, Al-Regaiey KA, Del Rosario Lim MM, Bonkowski MS, Panici JA, Przybylski GK & Bartke A 2005 Caloric restriction results in decreased expression of peroxisome proliferators-activated receptors (PPARs) superfamily in muscle of normal and long-lived GHR-KO mice. *Journal of Gerontology. Series A, Biological Sciences and Medical Sciences* **60A** 1238–1245.
- Mathews LS, Hammer RE, Brinster RL & Palmiter RD 1988 Expression of insulin-like growth factor I in transgenic mice with elevated levels of growth hormone is correlated with growth. *Endocrinology* **123** 433–437.
- Mayo KE, Hammer RE, Swanson LW, Brinster RL, Rosenfeld MG & Evans RM 1988 Dramatic pituitary hyperplasia in transgenic mice expressing a human growth hormone releasing factor gene. *Molecular Endocrinology* **2** 606–612.
- McGrane MM, Yun JS, Moorman AFM, Lamers WH, Hendrick GK, Arafah BM, Park EA, Wagner TE & Hanson RW 1990 Metabolic effects of

- developmental, tissue-, and cell-specific expression of a chimeric phosphoenolpyruvate carboxykinase (GTP)/bovine growth hormone gene in transgenic mice. *Journal of Biological Chemistry* **265** 22371–22379.
- Miquet JG, Sotelo AI, Bartke A & Tury D 2004 Suppression of growth hormone (GH) JAK2/STAT5 signaling pathway in transgenic mice overexpressing bovine GH. *Endocrinology* **145** 2824–2832.
- Miquet JG, Sotelo AI, Bartke A & Tury D 2005 Increased SH2-B β content and membrane association in transgenic mice overexpressing GH. *Journal of Endocrinology* **185** 301–306.
- Nicholson KM & Anderson NG 2002 The protein kinase B/Akt signaling pathway in human malignancy. *Cellular Signalling* **14** 381–395.
- Orian JM, Lee CS, Weiss LM & Brandon MR 1989 The expression of a metallothionein-ovine growth hormone fusion gene in transgenic mice does not impair fertility but results in pathological lesions in the liver. *Endocrinology* **124** 455–463.
- Orian JM, Tamakoshi K, Mackay IR & Brandon MR 1990 New murine model of hepatocellular carcinoma: transgenic mice expressing metallothionein-ovine growth hormone fusion gene. *Journal of the National Cancer Institute* **82** 393–398.
- Parsons JT 2003 Focal adhesion kinase: the first ten years. *Journal of Cell Science* **116** 1409–1416.
- Parsons SJ & Parsons JT 2004 Src family kinases, key regulators of signal transduction. *Oncogene* **23** 7906–7909.
- Perry JK, Emerald BS, Mertani HC & Lobie PE 2006 The oncogenic potential of growth hormone. *Growth Hormone and IGF Research* **16** 277–289.
- Quaife CJ, Mathews LS, Pinkert CA, Hammer RE, Brinster RL & Palmiter RD 1989 Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice. *Endocrinology* **124** 40–48.
- Ram P & Waxman DJ 2000 Role of the cytokine-inducible SH2 protein CIS in desensitization of STAT5b signaling by continuous growth hormone. *Journal of Biological Chemistry* **275** 39487–39496.
- Ram PA, Park SH, Choi HK & Waxman DJ 1996 Growth hormone activation of STAT1, STAT3, and STAT5 in rat liver. *Journal of Biological Chemistry* **271** 5926–5940.
- Schlaepfer DD & Mitra SK 2004 Multiple connections link FAK to cell motility and invasion. *Current Opinion in Genetics and Development* **14** 92–101.
- Silva CM 2004 Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene* **23** 8017–8023.
- Snibson KJ 2002 Hepatocellular kinetics and the expression of growth hormone (GH) in the livers and liver tumours of GH-transgenic mice. *Tissue and Cell* **34** 88–97.
- Snibson KJ, Bhathal PS, Hardy CL, Brandon MR & Adams TE 1999 High, persistent hepatocellular proliferation and apoptosis precede hepatocarcinogenesis in growth hormone transgenic mice. *Liver* **19** 242–252.
- Song G, Ouyang G & Bao S 2005 The activation of Akt/PKB signaling pathway and cell survival. *Journal of Cellular and Molecular Medicine* **9** 59–71.
- Sotelo AI, Dominici FP, Engbers C, Bartke A, Talamantes F & Tury D 1995 Growth hormone binding protein (GHBP) in normal mice and in transgenic mice expressing bovine growth hormone gene. *American Journal of Physiology* **268** E745–E751.
- Sotelo AI, Bartke A, Kopchick JJ, Knapp JR & Tury D 1998 Growth hormone (GH) receptors and binding proteins and IGF-I concentrations in the serum of transgenic mice expressing bovine GH agonist or antagonist. *Journal of Endocrinology* **158** 53–59.
- Sotelo AI, Miquet JG, González L, Bartke A & Tury D 2008 Vitamin D3 cannot revert desensitization of growth hormone (GH)-induced STAT5-signaling in GH-overexpressing mice non-calcemic tissues. *Growth Hormone and IGF Research* **10** 148–156.
- Thomas SM & Brugge JS 1997 Cellular functions regulated by Src family kinases. *Annual Review of Cell and Developmental Biology* **13** 513–609.
- Thomas MB & Zhu AX 2005 Hepatocellular carcinoma: the need for progress. *Journal of Clinical Oncology* **23** 2892–2899.
- Uytendaele I, Lemmens I, Verhee A, De Smet AS, Vandekerckhove J, Lavens D, Peelman F & Tavernier J 2007 MAPPIT analysis of STAT5, CIS and SOCS2 interactions with the growth hormone receptor. *Molecular Endocrinology* **21** 2821–2831.
- Wang Z, Masternak MM, Al-Regaiey KA & Bartke A 2007 Adipocytokines and the regulation of lipid metabolism in growth hormone transgenic and calorie-restricted mice. *Endocrinology* **148** 2845–2853.
- Waters MJ, Hoang HN, Fairlie DP, Pelekanos RA & Brown RJ 2006 New insights into growth hormone action. *Journal of Molecular Endocrinology* **36** 1–7.
- Woelfle J & Rotwein P 2004 *In vivo* regulation of growth hormone-stimulated gene transcription by STAT5b. *American Journal of Physiology. Endocrinology and Metabolism* **286** E393–E401.
- Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, Wada M, Honjo M, Takahashi M, Takahashi T, Matsuda T *et al.* 1997 Tyrosine phosphorylation of the EGF receptor by the kinase JAK2 is induced by growth hormone. *Nature* **390** 91–96.
- Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, Wada M, Honjo M, Takahashi M, Takahashi T, Hirai H *et al.* 1998 Growth hormone-induced tyrosine phosphorylation of EGF receptor as an essential element leading to MAP kinase activation and gene expression. *Endocrine Journal* **45** 27–31.
- Zhu T, Goh EL, Graichen R, Ling L & Lobie PE 2001 Signal transduction via the growth hormone receptor. *Cellular Signalling* **13** 599–616.
- Zhu T, Ling L & Lobie PE 2002 Identification of a JAK2-independent pathway regulating growth hormone (GH)-stimulated p44/42 mitogen-activated protein kinase activity. GH activation of Ral and phospholipase D is Src-dependent. *Journal of Biological Chemistry* **277** 45592–45603.

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