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Growth hormone (GH)/STAT5 signaling during the growth period in liver of mice overexpressing GH

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Abstract

Growth hormone (GH)/STAT5 signaling is desensitized in liver of adult transgenic mice overexpressing GH; however, these animals present greater body size. To assess if the STAT5 pathway is active during the growth period in liver of these animals, and how signaling modulators participate in this process, growing transgenic mice and normal siblings were evaluated. STAT5 does not respond to an acute GH-stimulus but presents higher basal phosphorylation in liver of growing GH-overexpressing mice. GH receptor and positive modulators GR and HNF1 display greater abundance in transgenic animals, supporting STAT5 activity. Negative modulators CIS and PTP1B are increased in GH-overexpressing mice. Suppressors SOCS2 and SOCS3 exhibit higher mRNA levels in transgenic mice but lower protein content, suggesting they are being actively degraded. Therefore, STAT5 signaling is increased in liver of GH-transgenic mice during the growth period, with a balance between positive and negative effectors resulting in an accelerated but controlled growth.

Keywords

GH signaling; STAT5; GH-overexpressing mice; growth; liver

1. INTRODUCTION

Growth hormone (GH) is involved in the promotion of skeletal growth and in a variety of metabolic functions; its actions are accomplished both directly and indirectly by the induction of insulin-like growth factor 1 (IGF1). Liver is a primary target organ for GH and the major site of production of circulating IGF1, the principal mediator of the somatotropic function. GH binding to a growth hormone receptor (GHR) dimer leads to cross-activation

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of receptor-associated tyrosine-kinase JAK2, which phosphorylates GHR on multiple intracellular tyrosine residues (Brooks and Waters 2010; Sedek, et al. 2014). These residues become anchoring sites for several signaling mediators, among which signal transducer and activator of transcription 5b (STAT5b) plays a major role in GH action. GH-dependent STAT5 activation is essential for postnatal body growth, in line with its participation in GH-induced IGF1 gene transcription in the liver (Barclay, et al. 2010; Rotwein 2012; Woelfle, et al. 2003).

Down-regulation or termination of the signal is attained by blockade or remotion of phosphotyrosines at the GHR-activated complex (Lanning and Carter-Su 2006). Protein tyrosine phosphatases (PTPs), like PTP1B, PTPH1 and the cytosolic SH2 containing protein-tyrosine phosphatases (SHP) 1 and 2, interact with GHR, JAK2 and nuclear tyrosine-phosphorylated STAT5b in a GH-dependent manner (Flores-Morales, et al. 2006; Lanning and Carter-Su 2006; Pilecka, et al. 2007). In addition, GH induces the expression of suppressors of cytokine signaling (SOCS)/cytokine-induced suppressor (CIS) proteins, CIS, SOCS1, SOC2 and SOCS3 via the STAT5 pathway, albeit with different kinetics (Adams, et al. 1998; Tollet-Egnell, et al. 1999). SOCS1 and 3 inhibit JAK2 kinase activity, while CIS and SOCS2 bind to phosphotyrosine residues on the carboxy-terminus of GHR, thus interfering with recruitment of signaling mediators. Moreover, these suppressors drive signaling complexes to proteasome degradation (Kile, et al. 2002; Linossi and Nicholson 2012). In the liver, SOCS2 expression is also induced by the fasting-induced hormone, fibroblast growth factor 21 (FGF21), resulting in the inhibition of STAT5 (Inagaki, et al. 2008). The influence of FGF21 on body growth is evident in transgenic mice overexpressing this factor, which exhibit reduced growth, whereas targeted-disruption of FGF21 blunts food-restriction impairment of growth (Inagaki et al. 2008; Kubicky, et al. 2012). Another protein involved in the regulation of STAT5 activity is BCL6, a strong transcriptional repressor that binds to DNA motifs distinct but overlapping with those of STAT5, thus preventing its action. In liver, BCL6 binds preferentially to female-specific GH-regulated genes, precluding their transcription (Zhang, et al. 2012).

Several transcription factors modulate STAT5 binding to DNA, including the glucocorticoid receptor (GR) and the hepatocyte nuclear factor 1 α (HNF1α). Liver-specific inactivation of these positive modulators is associated with reduced somatic growth in mice (Engblom, et al. 2007; Lin, et al. 2008; Tronche, et al. 2004). GR is a STAT5 coactivator in the liver (Engblom et al. 2007; Tronche et al. 2004), whereas HNF1α regulates GR expression to control postnatal body growth, indirectly modulating STAT5 (Lin et al. 2008).

Rodents present two phases of rapid growth. The first is independent of GH and occurs immediately after birth. The second is GH-dependent and begins soon after the second week of life (Liu and LeRoith 1999; Lupu, et al. 2001; Tang, et al. 2005), denoting the transition of the autocrine/paracrine control of growth to the central regulation (Hyatt, et al. 2004). In accordance, transgenic mice overexpressing GH exhibit accelerated growth from the third week of age, in spite of having high circulating GH levels since birth (Mathews, et al. 1988; McGrane, et al. 1990). As a result, they achieve greater adult body size than control mice. At adulthood, these animals present GH-induced STAT5-signaling desensitization in the liver, as it does not become phosphorylated upon an acute GH-stimulus. Moreover, basal levels of

hepatic STAT5 phosphorylation in adult transgenic mice are comparable to those of normal animals (Gonzalez, et al. 2002; Miquet, et al. 2004; Sotelo, et al. 2008). It is therefore the aim of the present work to study if this pathway is activated differentially at earlier ages, i.e., during the GH-dependent phase of growth. For this purpose, three ages were selected, one before and one after the growth burst (2 and 4-week-old animals, respectively) and the young adult (9-week-old control). The current work presents an *in vivo* study of STAT5-mediated GH-signaling in liver of growing GH-overexpressing mice, since this pathway is directly related to IGF1 induction and longitudinal body growth. Results will contribute to understanding how the organism adapts to an excess of a hormone.

2. MATERIALS AND METHODS

2.1. Animals

Transgenic PEPCK-bGH mice containing the bGH gene fused to control sequences of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene were derived from animals kindly provided by Dr. Thomas E. Wagner and Jeung S. Yun (Ohio University, Athens, OH). Hemizygous transgenic mice were produced by mating transgenic males with normal C57BL/6 X C3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME). Normal siblings of transgenic mice were used as controls. The mice were housed three to five per cage in a room with controlled light (12 h light per day) and temperature (22 ± 2 C). The animals had free access to food (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.

Three cohorts of animals were used. The first one was used to determine body growth at different age points. The second was used for GH-stimulation studies, while the third was used to determine content of GH-signaling mediators as well as tibial length. In these last two cohorts, animals were 14, 28 and 63 days of age (referred to as 2, 4 and 9 weeks old). For GH-stimulation studies, female mice received 5 μg oGH per gram of body weight (ovine GH, obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA) or saline solution by intraperitoneal administration 7.5 minutes before necropsy. Animals were killed by cervical dislocation under isofluorane anesthesia, and livers were removed and stored at −80° C until use.

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 institutional regulations. Experiments were approved by SIU Laboratory Animal Care Committee.

2.2. Liver solubilization and immunoblotting

Liver solubilization, CIS immunoprecipitation and preparation of samples for immunoblotting (IB) have been previously described (Martinez, et al. 2013). For IB, each lane was loaded with 40 μg of protein from whole tissue solubilizates. After blotting and blocking of PVDF membranes, they were incubated overnight at 4°C with antibodies anti-BCL6 1:1.000 (αBCL6; C-19, No. 368), anti-CIS 1:200 (αCIS; N-19,No. 1529), anti-FGF21 1:1.000 (αFGF21; V-16, No. 16842), anti-GR 1:300 (αGR; M-20, No. 1004), anti-HNF1

1:300 (αHNF1; H-205, No. 8986), anti-MUP 1:2.000 (αMUP, FL-180, No. 66976), anti-SOCS2 1:600 (αSOCS2; H-74,No. 9022), anti-SOCS3 1:300 (αSOCS3; H-103, No. 9023), anti-STAT5 1:10.000 (αSTAT5; C-17, No. 835), purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA); anti-SHP1 1:1000 (αPTP1C/SHP1, No. 610126), anti-SHP2 1:1000 (αPTP1D/SHP2, No. 610622), obtained from BD Transduction Laboratories (Franklin Lakes, NJ, USA); anti-phospho-STAT5a/b (Y694/Y699) 1:1.000 (αpSTAT5a/b, No.05-495), anti-PTP1B 1:2.500 (αPTP1B, No. 07-088), purchased from Millipore Corporation (Billerica, MA, USA); or antibody anti-GHR anti serum (αGHR) 1:1000, generously provided by Dr. S.J. Frank (Zhang, et al. 2001). Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL-Plus, Amersham, GE Healthcare LifeSciences) using hyperfilm ECL (GE Healthcare LifeSciences), and band intensities were quantified using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Antibody dilutions were selected based on previous reports, or according to recommendations of the providers; in each case, the bands to be quantified fell within the linear range of the method.

Immunoblotting experiments were designed to compare three variables in parallel: age, genotype and stimuli or age, genotype and sex. Each experimental set contained different individuals; thus, n, the number of individuals assayed per group, also represents the number of experiments. Average of values obtained for 9-week-old male mice was used as reference (100% value). Results are presented as mean ± standard error (SEM) of the number of samples indicated.

2.3. Protein loading control

Protein loading control could not be performed by immunoblotting detection of standard proteins in the liver for this GH-overexpressing mouse model. Actin exhibited age and genotype-dependent variation in hepatic protein abundance, whereas tubulin content did not show uniformity between genotypes (Supplementary Figure 1A) (Martinez et al. 2013; Miquet, et al. 2013). Therefore, the following determinations were routinely performed. Prior to immunoblotting (IB), homogeneity of sample preparation was assessed by SDS-PAGE followed by Coomassie blue staining. During IB, Ponceau staining was used to evaluate goodness of electrotransference (Torre, et al. 2011). After immunoblotting experiments, protein loading control was performed by Coomassie blue staining (CBS) of the PVDF membranes (Welinder and Ekblad 2011).

2.4. Real-time reverse transcriptase PCR

Total hepatic RNA was extracted using the phenol chloroform method. cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad), and the relative expression of the genes was analyzed by quantitative RT-PCR (qRT-PCR) as previously described (Ramirez, et al. 2010). Table 1 shows the sequence of the primer sets used, obtained from Invitrogen.

This method was also used for genotyping transgenic mice, since they cannot be distinguished from their normal siblings by size at 2 weeks of age. The sequences used to determine the expression of the transgene are described in Table 1.

2.5. Statistical analysis

Statistical analysis was performed using the GraphPad Prism statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Data were first analyzed by one-way ANOVA followed by Bonferroni to evaluate stimuli or sex differences. Since no significant differences were found for protein or mRNA content between sexes for every age and genotype analyzed, results were presented in separate bar charts for each sex. Subsequently, data were analyzed by two-way ANOVA and Bonferroni post test to assess differences by age and genotype. Data were considered statistically significant if $P < 0.05$.

3. RESULTS

3.1. Body growth

Body weight was determined and the expected two phases of rapid growth were observed, separated by a small pause between days 14 and 16. Male mice exhibited higher body weight than females. In normal mice, this difference was detected beginning at the fourth week of age; while, in transgenic animals, the difference between sexes was less pronounced. Transgenic mice weight was significantly different from that of non-transgenic siblings starting at day 19 (Fig. 1A). When growth rate was assessed, more noticeable differences were observed between 2 and 3 weeks of age (Fig. 1A inserts).

In order to evaluate GH signaling in liver at different growth periods, representative ages were chosen: two weeks (2w), when animals of both genotypes still exhibit the same body weight; four weeks (4w), when transgenic mice display greater body size than normal siblings; and nine weeks (9w), young adults, which were considered as reference. The selected ages are shown with arrows in Fig. 1A. For the selected ages, tibial length was determined. The extension of the tibias increased with age both for normal and transgenic mice, but the increase was larger for GH-overexpressing animals. When tibial length was assessed by genotype, greater values were obtained for 9-week-old transgenic mice compared to controls (Fig. 1B).

3.2. GHR gene expression and protein abundance

GH binding to its receptor is the first step in GH signaling events. It is already established that GH induces GHR expression in liver and, accordingly, it is augmented in GH-transgenic adult animals (Gonzalez, et al. 2007; Miquet et al. 2004). GHR levels have also been reported to increase with age (Maes, et al. 1983; Martinez et al. 2013; Mathews, et al. 1989). Moreover, it has been proposed that the magnitude of GH responsiveness correlates with GHR expression (Jiang, et al. 2007). Therefore, it was of interest to determine its content in growing GH-overexpressing mice.

Transgenic animals presented significantly higher GHR protein abundance than normal siblings since the fourth week of age (Fig. 1C–D). For GH-overexpressing mice, values did not exhibit a gradual increase with age, but presented a maximum at 4 weeks in both sexes (Fig. 1C–D). To detect if there was any age difference in normal animals, samples had to be assessed separately, since high GHR content in transgenic mice concealed results. Non-transgenic littermates exhibited an age-dependent profile similar to that in GH-

overexpressing animals, although the rise at 4 weeks was not as pronounced (Supplementary Figures 1A–B). *Ghr* mRNA expression was higher for transgenic animals at every age studied, indicating the high protein content these mice exhibit correlates with elevated receptor gene expression in these animals (Fig. 1E–F).

3.3. STAT5 activation

3.3.1 STAT5 phosphorylation upon an exogenous stimulus—To study GH sensitivity during the growth period, animals received a GH stimulus or saline for 7.5 minutes, after which livers were collected and solubilized. Tyrosine-phosphorylation of STAT5 proteins was determined by immunoblotting with a specific antibody against the activating residue in STAT5 a and b (pSTAT5). Assays were restricted to female mice to assess age and genotype simultaneously. Normal mice responded at every age studied, exhibiting maximal STAT5 phosphorylation levels at 2 weeks of age, and declining thereafter (Fig. 2A). On the other hand, transgenic mice did not respond to the stimulus at any age. Since we have already assessed GH response to an exogenous stimulus in non-transgenic growing mice and found no sex difference at any age (Martinez et al., 2013), and taking in account that GH secretion is not sexually dimorphic in transgenic mice (presented in section 3.6), we would expect GH desensitization at every age in GH-transgenic male mice as well. For this reason, and due to the high number of animals required, we preferred not to carry out the experiment in male mice.

3.3.2 Endogenous STAT5 activation—In normal mice, basal STAT5 phosphorylation levels displayed an increasing trend with age. GH-transgenic mice exhibited a different age-dependent pattern, presenting comparable pSTAT5 levels for 2- and 9-week-old animals and a two-fold increase at 4 weeks. When genotype difference was analyzed, GH-overexpressing growing mice achieved higher basal phosphorylation levels than control siblings (Fig. 2B–C). At 9-weeks of age, pSTAT5 did not exhibit difference by genotype for female mice, while transgenic males presented lower levels than their controls. This can be attributed to comparable pSTAT5 levels observed in transgenic animals for both sexes, whereas normal male mice exhibited greater levels than females, in accordance with recent observations (Miquet et al. 2013).

There is a slight discrepancy between basal STAT5 phosphorylation levels in Fig. 2A and 2B, this may be due to the masking of the non-stimulated signal in Fig. 2A, since the intensity of the endogenous signal is much weaker than that of the stimulated one.

3.3.3 STAT5 protein content and phosphorylation/protein content relation—When STAT5a/b protein content was assayed, 2-week-old mice exhibited the highest values, with greater levels in normal animals, whereas 4- and 9-week-old mice presented similar abundance in both genotypes (Fig. 2E–F).

GH-induced STAT5 phosphorylation values were related to STAT5 protein abundance. STAT5 protein content was determined for non-stimulated mice only (results not shown), since phosphorylated STAT5 migrates differentially in electrophoresis, yielding an unexpected increase in immunoblotting intensity that may distort the results (Martinez et al. 2013; Thangavel and Shapiro 2007). For saline-treated mice and for GH-stimulated

transgenic animals, transformed values closely resembled the original ones, whereas for GH-stimulated normal mice, similar results were obtained at every age studied, indicating that the pronounced GH-induced activation in 2-week-old mice is due to higher protein content at that age (Fig. 2D).

3.3.4 IGF1: downstream endogenous STAT5 activation—To further address this pathway, the hepatic expression of IGF1, a STAT5-mediated GH-induced gene and the principal mediator of GH action on somatic growth, was evaluated. Transgenic mice exhibited increased hepatic IGF1 gene expression at every age studied compared to normal controls (Fig. 2E–F). Consistent with STAT5 basal phosphorylation levels, during the growth period there was an age-dependent increase in IGF1 transcription levels in transgenic animals. However, 4- and 9-week-old GH-overexpressing mice exhibited comparable transcript levels, despite the different STAT5 phosphorylation levels they present. Furthermore, in adult mice, IGF1 transcript expression does not seem to correlate with STAT5 phosphorylation status.

3.4. Phosphatase protein content

GH signaling is limited by dephosphorylation of activated mediators by specific phosphatases. Hepatic content of these enzymes was assayed, and no significant age, sex, or genotype differences were found for SHP1 and SHP2 proteins (Fig. 3A–D). On the other hand, transgenic mice presented higher PTP1B levels compared to normal siblings at every age studied, with a maximum at 4 weeks for both sexes (Fig. 3E–F). Since we had previously reported age differences in PTP1B levels in normal mice for another strain, samples from non-transgenic animals were run separately to assess age differences. Maximal PTP1B abundance was found at 2 weeks of age and declined thereafter (Supplementary Figure 1C–D), in agreement with previous observations (Martinez et al. 2013).

3.5. SOCS protein content and gene expression

CIS, SOCS2 and SOCS3 are suppressors of cytokine signaling induced by GH in the liver; therefore, their hepatic content and gene expression were evaluated in growing GH-overexpressing mice by immunoblotting and quantitative RT-PCR, respectively.

Transgenic mice had increased liver CIS expression at every age studied compared to normal siblings, both for protein and mRNA content (Fig. 4A–D). For protein abundance, highest CIS levels were observed in 4-week-old GH-overexpressing animals. In normal mice, CIS content was almost undetectable when samples were run next to those of transgenic animals; therefore, they were assessed separately. Non-transgenic 2-week-old animals exhibited higher CIS levels, while 4- and 9-week-old animals had similar abundance (Supplementary Figure 1E–F) in accordance with previous observations in another mouse strain (Martinez et al. 2013). When assessed by age, *Cis* mRNA content was higher at 2 weeks and similar at 4 and 9 weeks both in normal and transgenic mice, closely resembling the protein profile in normal mice (shown in Supp. 1E–F). Differences were less pronounced in transgenic female animals.

SOCS3 protein content was lower in transgenic mice than in normal animals at every age studied. Normal mice presented an age-dependent pattern, with lower values at 2 weeks, which gradually increased to achieve adult values. Transgenic mice, on the contrary, presented almost undetectable levels of the suppressor at every age evaluated (Fig. 5A–B). Hepatic SOCS2 protein content displayed a similar pattern; normal mice exhibited a gradual increase with age, whereas transgenic animals exhibited uniform values that were lower than those of 2-week-old normal animals (Fig. 5C–D). Since SOCS3 and SOCS2 are induced by GH, like CIS, their protein content in transgenic mice would have been expected to be higher or at least the same as in normal animals, rather than lower. To elucidate this controversy, SOCS3 and SOCS2 gene expression was assessed. *Socs3* mRNA hepatic content was numerically higher in GH-overexpressing animals than in normal siblings at every age studied, genotype statistical significance was achieved for 9-week-old animals. Moreover, *Socs3* mRNA content exhibited an age-dependent increment for both genotypes (Fig. 5E–F). *Socs2* mRNA expression was also higher in GH-overexpressing animals than in control siblings at every age studied. However, whereas hepatic *Socs2* mRNA content displayed a declining tendency as a function of age for normal animals, GH-transgenic mice presented a different scenario, with higher values at 2 weeks of age, compared to 4-week-old animals or the young adult (Fig. 5G–H).

3.6. STAT5 modulators abundance

Hepatic STAT5 activity is also inhibited by FGF21 and BCL6. FGF21 protein content did not vary with age in normal mice of both sexes, while it was high in 2-week-old transgenic animals and declined thereafter, attaining normal animal levels (Fig. 6A–B). BCL6 displayed a trend to increasing levels during the growth period in both normal and transgenic mice, with statistical significance for GH-overexpressing males only. Moreover, transgenic animals exhibited numerically higherBCL6 levels than their normal siblings at 4 and 9 weeks of age (Fig. 6C–D)

BCL6 has been reported to be suppressed by growth hormone (Chen, et al. 2009; Meyer, et al. 2009). In GH-transgenic mice the hormone is produced in extrapituitary tissues; thus, secretion in these animals is not centrally regulated, rendering a continuous pattern of circulating GH. Therefore, the GH-secretory status of these animals was assessed by determination of an indirect marker, the major urinary proteins (MUPs). MUP expression and secretion are regulated by the intermittent secretion of GH and can be used as pulsatility markers of the somatotropic axis. MUPs were detected only in normal mice of 4 and 9 weeks of age, after the onset of puberty, with higher levels in males than in females, as expected (Fig. 6E). These results are consistent with the continuous GH secretory pattern of transgenic mice.

STAT5 activity is positively modulated in the liver by glucocorticoid receptor (GR) and hepatic nuclear factor 1 (HNF1). Normal and transgenic mice displayed a similar age-related GR pattern: low levels at 2 weeks, maximal values at 4 weeks and slightly lower levels in adulthood (Fig. 6F–G). From the fourth week onward, GH-overexpressing mice exhibited higher content than non-transgenic siblings in female mice. For HNF1, the same age profile

was observed (Fig. 6H–I). Transgenic mice had higher levels at every age, which achieved significant difference at 4 weeks.

4. DISCUSSION

Previous reports from our laboratory revealed the inability of adult GH-overexpressing giant mice to respond to a GH stimulus, both in different tissues and in different GH-transgenic lines. Moreover, basal STAT5 phosphorylation in those GH-overexpressing adults was not higher than in their non-transgenic littermates, although these animals have greatly elevated circulating GH levels (Gonzalez et al. 2002; Miquet et al. 2004; Sotelo et al. 2008). Current results show GH-induced STAT5 insensitivity at every age studied in GH-transgenic mice. However, there is an age-dependent variation for basal STAT5 activation, which is higher during the growth hormone-dependent phase of growth, suggesting this pathway is involved in the accelerated growth these mice exhibit. After this initial finding, the next goal was to elucidate the contribution of different modulators of the GH-STAT5 signaling pathway in order to dissect the mechanisms that might account for differences between normal and transgenic mice involved in the regulation of this pathway during the growth period.

Growth hormone receptor levels are low during fetal and early postnatal periods, probably contributing to the lack of GH influence on somatic growth in the perinatal stage (Jiang et al. 2007). GH itself up-regulates GHR expression in the liver (Gonzalez et al. 2007; Iida, et al. 2004; Maiter, et al. 1988), in fact, GHR has been shown to be induced by GH through STAT5 signaling in cattle (Jiang et al. 2007). In growing GH-overexpressing mice, GHR expression is slightly higher than in normal littermates at 2 weeks of age, but it is markedly increased at the fourth week of age, when these animals can be distinguished from their control siblings by their increased body size, coinciding with maximal STAT5 endogenous phosphorylation. However, although adult transgenic mice exhibit higher GHR abundance than controls, they do not have greater pSTAT5 levels, suggesting the existence of mechanisms that attenuate GH signaling in transgenic adults.

Negative regulation of GH-signaling may be achieved by different effectors. Fast-acting mechanisms include recruitment of protein tyrosine-phosphatases involved in the recycling of STATs proteins (Kornfeld, et al. 2008); in particular, PTP1B, SHP1 and SHP2 have been related to STAT5. SHP1 and SHP2 presented no significant age- or genotype-related differences, whereas PTP1B content was elevated in transgenic mice at all ages, suggesting this phosphatase may participate in GH-insensitivity by rapidly dephosphorylating activated substrates.

Other negative modulators of the GH-STAT5 pathway are the transcriptional feedback regulators, the suppressors of cytokine signaling, which are induced as early response genes. CIS was overexpressed in GH-transgenic animals and presented an age-dependent profile, suggesting this suppressor contributes to the mitigation of GH action at every age in GH-overexpression conditions. In fact, CIS has been regarded as the only factor down-regulating STAT5-mediated GH-signaling under continuous exposure to the hormone (Thangavel and Shapiro 2007). Like GHR, CIS reached higher levels at 4 weeks of age coinciding with the higher basal STAT5 phosphorylation found in transgenic animals. This is not unexpected,

since CIS has also been shown to be induced through STAT5 (Verdier, et al. 1998). Moreover, the parallel between the GH-induced age-variation for CIS and GHR is in line with CIS-mediated internalization of the receptor to limit GH-induced signaling (Landsman and Waxman 2005). In contrast, the hepatic protein content of the negative regulators SOCS3 and SOCS2 was lower in transgenic mice compared to normal siblings at every age studied. This behavior, although surprising since these suppressors are induced by GH, is in agreement with our previous observations in GH-overexpressing adult animals (Gonzalez et al. 2002; Miquet et al. 2004; Sotelo et al. 2008). SOCS2 was shown to bind to SOCS1 and SOCS3 and to target them for proteosomal degradation, therefore putting an end to the SOCS termination signal and allowing the resensitization of the JAK2/STAT5 pathway (Croker, et al. 2008; Flores-Morales et al. 2006; Piessevaux, et al. 2006; Tannahill, et al. 2005). When *Socs3* and *Socs2* mRNA levels were analyzed, higher expression was found in transgenic animals for both genes, indicating these suppressors are being effectively induced by the elevated GH levels. Therefore, even when *Socs3* and *Socs2* gene transcription is increased in transgenic mice, the protein content of these suppressors is decreased, suggesting they are being actively degraded, probably as a consequence of their own negative regulation. Moreover, SOCS2 has been shown to be a key regulator of GHR sensitivity, as it is part of an ubiquitin ligase complex that regulates growth hormone receptor levels (Vesterlund, et al. 2011).

Additionally, STAT5 activity is inhibited in the liver by FGF21 and BCL6. FGF21 is a hepatic hormone involved in energy homeostasis, it is induced by fasting and may participate in liver GH-activated STAT5-signaling down-regulation caused by food deprivation (Inagaki et al. 2008; Kliewer and Mangelsdorf 2010; Kubicky et al. 2012). Moreover, FGF21 expression has also been reported to be induced by GH through STAT5 in that tissue, indicative of the existence of another negative feedback loop to restrain GH-STAT5 signaling (Yu, et al. 2012). Our results in transgenic mice showed higher levels at 2 weeks of age, which do not coincide with the maximal basal STAT5 phosphorylation found at 4 weeks. However, FGF21 was proposed to inhibit STAT5 activation by promotion of SOCS2 expression (Inagaki et al. 2008), and the elevated levels of FGF21 in 2-week-old transgenic mice correlate with high values of *Socs2* mRNA found at that age. In accordance, STAT5 phosphorylation is low in 2-week-old transgenic animals, despite the high STAT5 protein content these mice present.

BCL6 is a strong transcriptional repressor of STAT5 activity, and in turn, STAT5 is a repressor of *Bcl6* expression. Complete suppression of *Bcl6* is achieved in female rat liver, related to the permanent low activation of STAT5 found in this sex, whereas male interpulse STAT5 deactivation, may be permissive to BCL6 expression (Chen et al. 2009; Meyer et al. 2009; Zhang et al. 2012). In mice, this dimorphism is not as pronounced (Meyer et al. 2009). Transgenic animals exhibit continuous GH levels in both sexes; however, BCL6 levels are higher than those of non-transgenic controls. Moreover, *Bcl6* mRNA levels were reported to increase after puberty only in male rats (Meyer et al. 2009), in contrast to our observations of a moderate increase with age in both sexes and genotypes. Altogether, these results suggest that BCL6 becomes involved in GH-signaling after sexual maturation in GH-overexpressing mice, in an attempt to reduce the female-specific expression of genes

promoted by STAT5 that would predominate in these animals due to the continuous GH profile.

Glucocorticoid receptor (GR) acts in synergism with STAT5 in liver to induce the expression of GH-responsive genes related to body growth (Engblom et al. 2007; Tronche et al. 2004). GR protein content displayed an age-dependent pattern with a maximum at 4 weeks for both genotypes, suggesting its active participation in the growth phenomenon. However, this pattern was not observed in another mouse strain, the Swiss-Webster (Martinez et al, unpublished observations). Hepatocyte nuclear factor 1α (HNF1α) is another transcription factor required for postnatal growth, which regulates GR transcription activity, indirectly mediating STAT5 action (Lee, et al. 1998; Lin et al. 2008). In liver of growing mice, HNF1α had the same age-related profile as GR, with increased levels in transgenic mice as well. HNF1α is not directly regulated by GH; however, GH triggers a network of transcription factors that culminates in HNF1α activation, which has been reported in several species (Eleswarapu and Jiang 2005; Lahuna, et al. 2000; Rastegar, et al. 2000).

The relevance of hepatic GHR/STAT5 signaling in body growth has been questioned (Kaplan and Cohen 2007; Klover and Hennighausen 2007). Moreover, recent evidence points to the involvement of this signaling pathway in lipid metabolism in this organ (Barclay, et al. 2011; Fan, et al. 2009; Mueller, et al. 2011). However, these effects do not need to be mutually exclusive, and there is strong support for the implication of the GH/GHR/STAT5/IGF1 axis in liver in somatic growth. Studies with hepatic deletion of STAT5, GR or HNF1 show the interrelation of these three factors in the growth outcome (Engblom et al. 2007; Lin et al. 2008; Mueller, et al. 2012; Tronche et al. 2004). Moreover, liver STAT5 deletion in GH-overexpressing mice abolished the giant phenotype of the latter (Friedbichler, et al. 2012); JAK2 hepatic deletion is also associated with reduced size (Shi, et al. 2012). Liver specific deletion of the GHR in mice yielded animals with diminished adult body weight and length (List, et al. 2014). Results presented here indicate that GH participates in the molecular mechanisms that regulate the second phase of rapid growth before the manifestation of growth changes, i.e., before the third week of life, suggesting the intracellular machinery is set in action before growth. Our results are in agreement with the initial characterization of GH-transgenic models, in which hepatic IGF1 was shown to be up-regulated as early as the second week of life (Mathews et al. 1988). These authors had also observed the persistency of high IGF1 levels at adulthood in that GH-overexpression model (Mt-bGH mice) and concluded animals were already maximally stimulated. More recently, IGF1 has been reported not to be a predictable marker of GH function in mice (Bielohuby, et al. 2011), which may account for the lack of correlation we found between basal STAT5 phosphorylation levels and IGF1 induction in adult animals.

In summary, even though the principal GH signaling pathway STAT5 is desensitized in the liver of GH-overexpressing mice, it is basally active during the growth period and may be involved in the augmented growth these mice exhibit. Accompanying this higher basal STAT5 activation, there is a change in GH-signaling modulators directly or indirectly regulated by the hormone through the same pathway. Thus, GHR and coactivators GR and HNF1, as well as negative regulators such as CIS and PTP1B, are up-regulated in GH-

transgenic mice, especially during the GH-dependent phase of growth. SOCS2 and SOCS3, on the contrary, present lower protein levels in GH-overexpressing mice than in non-transgenic littermates; however, their gene expression was up-regulated in these animals, suggesting they are involved in active degradation to modulate GH signal. Thus, STAT5 signaling is enhanced during the growth period in GH-overexpressing mice liver, when transgenic mice exhibit accelerated growth, revealing a counterbalance between up-regulation of positive and negative modulators that later in life seem to restrain basal STAT5 activation and to bring it close to normal levels. Moreover, the up-regulation of negative modulators of STAT5 activation may account for the lack of sensitivity towards the GH-exogenous stimulus found for these GH-overexpressing mice at every age studied.

Supplementary Material

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Acknowledgments

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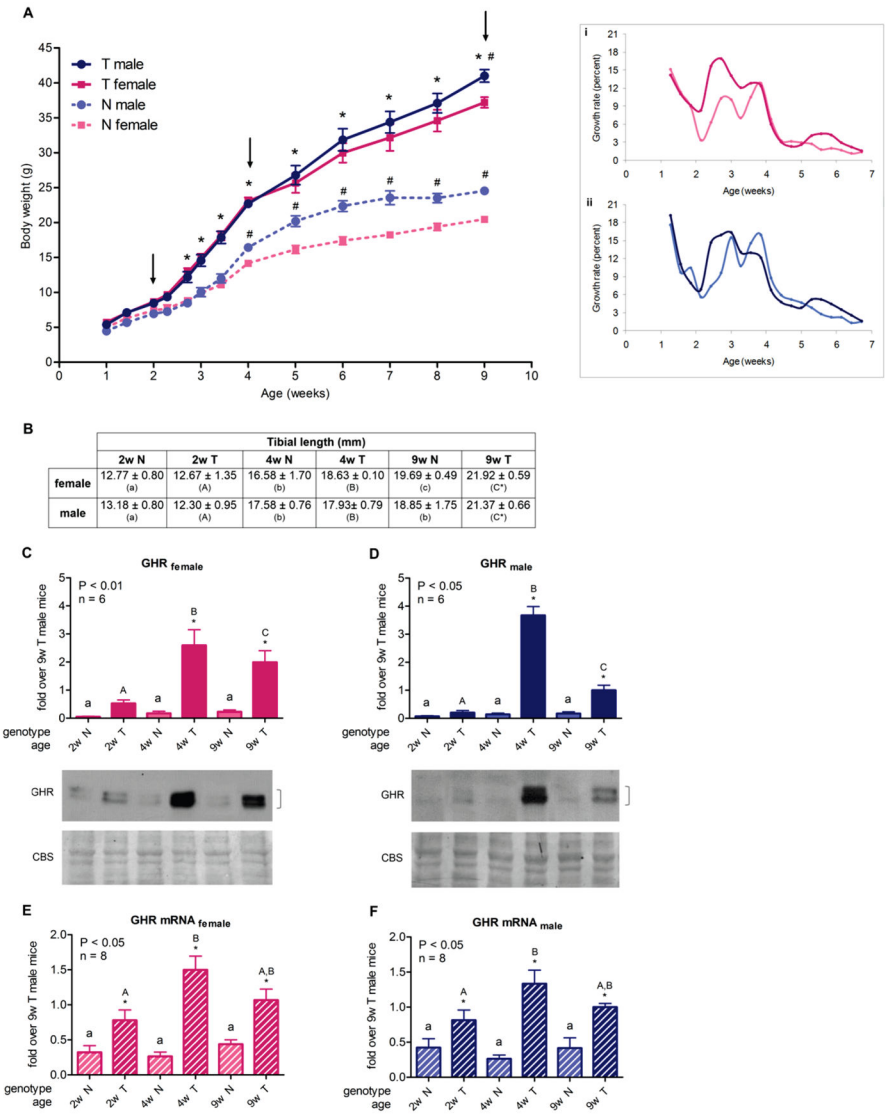

Fig. 1.

Body growth and GHR hepatic expression in growing GH-transgenic mice and normal littermates. A. Body weight of GH-overexpressing mice and normal littermates; inserts: Body growth rate in female mice (i) and male mice (ii). B. Tibial length of GH-overexpressing mice and normal littermates. C. GHR hepatic protein content in female mice; D. GHR hepatic protein content in male mice; E. GHR mRNA hepatic expression in female mice; F. GHR mRNA hepatic expression in male mice. Body weight and tibial length were assessed for transgenic (T) and normal (N) female and male mice.

Body growth rate was estimated as percent of body weight increment every two days. Data are the mean ± SEM of 10 (A) or 7 (B) samples per group, each one representing a different animal. Asterisks indicate significant difference between GH-overexpressing animals and their corresponding non-transgenic age-control, number signs indicate significant difference between females and males of the same genotype. Arrows indicate selected working ages.

Different letters denote significant difference by age; small letters correspond to normal mice and capital letters to transgenic animals.

To determine protein abundance, equal amounts of solubilized liver protein were assessed by immunoblotting. Coomassie blue staining (CBS) of PVDF membrane is shown. Bands were quantified by scanning densitometry. To determine gene expression, mRNA was assessed by qRT-PCR from total RNA extracts. Values were expressed as the fold increase over 9-week-old transgenic male mice. Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote significant difference by age; small letters correspond to normal mice and capital letters to transgenic animals. Asterisks indicate significant difference between GH-overexpressing animals and their corresponding non-transgenic age controls. Square brackets show the quantified bands.

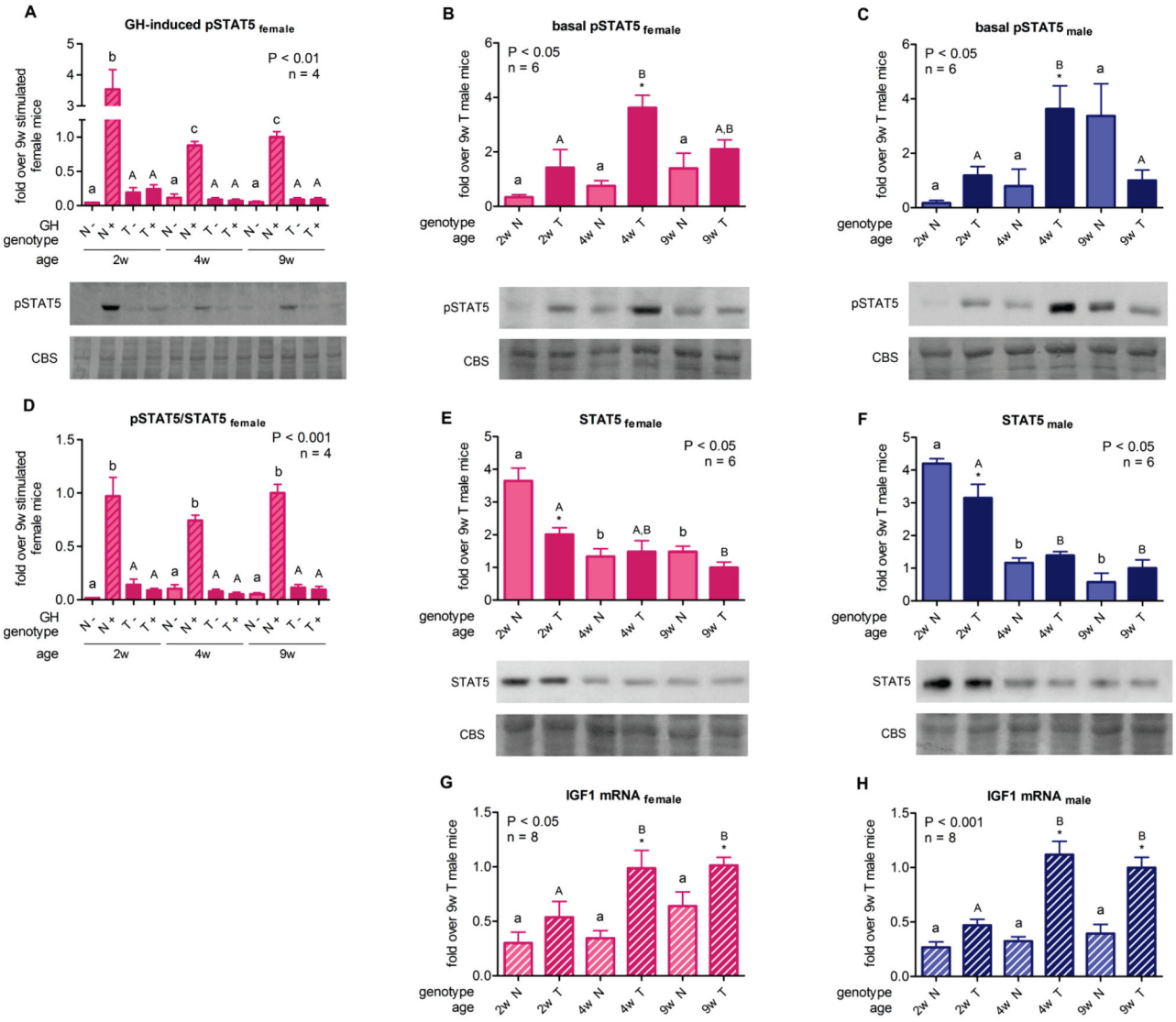

Fig. 2.

STAT5 signaling in liver of growing GH-overexpressing mice and normal littermates. A. GH-induced STAT5-tyrosine phosphorylation in female mice; B. Basal STAT5-tyrosine phosphorylation in female mice; C. Basal STAT5-tyrosine phosphorylation in male mice; D. Tyrosine phosphorylation/protein content relation for GH-induced STAT5 phosphorylation; E. STAT5 protein abundance in female mice; F. STAT5 protein abundance in male mice; G. IGF1 mRNA hepatic expression in female mice; H. IGF1 mRNA hepatic expression in male mice.

Equal amounts of solubilized tissue were analyzed by immunoblotting; Coomassie blue staining (CBS) of PVDF membrane is shown. mRNA was assessed by qRT-PCR from total RNA extracts. Results were expressed as the fold increase over the mean value measured for GH-stimulated 9-week-old normal female mice (A,D) or as the fold increase over 9-week-old transgenic male mice (B–H). Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote

significant difference by age; small letters correspond to normal mice and capital letters to transgenic animals. Asterisks indicate significant difference between GH-overexpressing animals and their corresponding non-transgenic age controls.

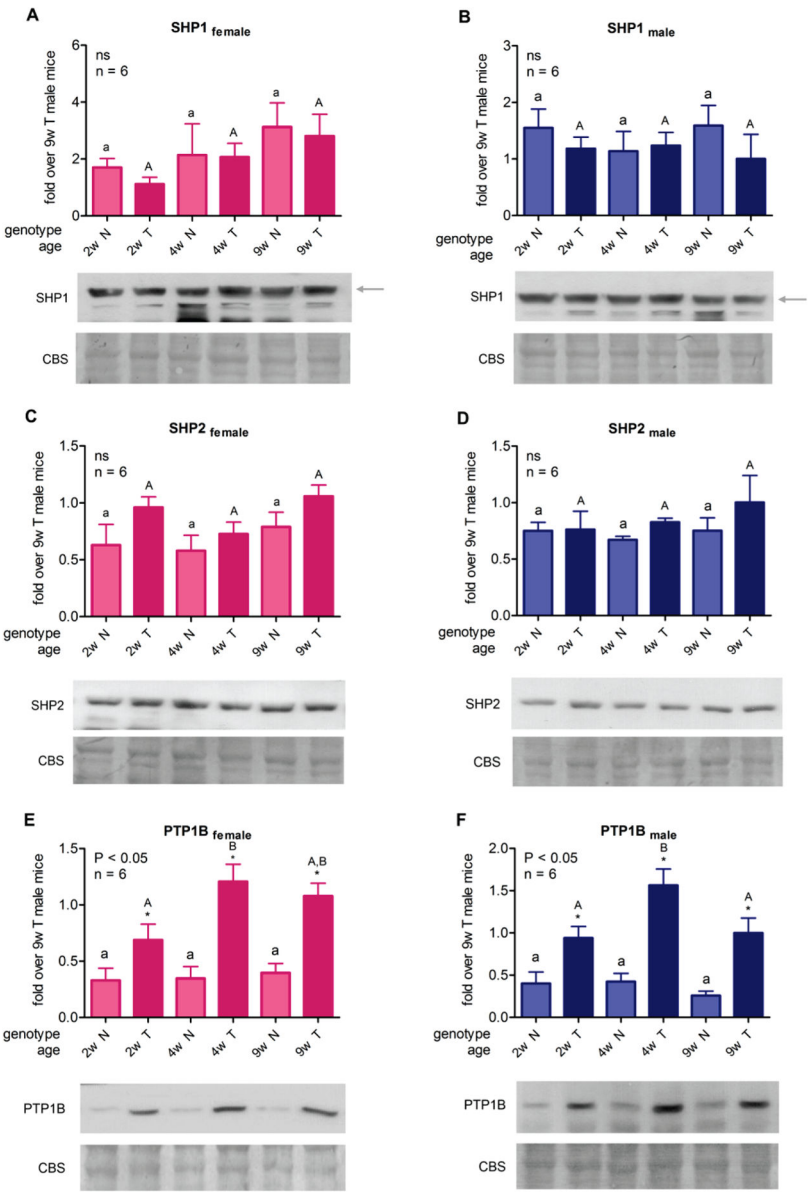

Fig. 3.

Phosphatase protein abundance in liver of growing GH-overexpressing mice and normal littermates. A. SHP1 content in female mice; B. SHP1 content in male mice; C. SHP2 content in female mice; D. SHP2 content in male mice; E. PTP1B content in female mice; F. PTP1B content in male mice.

Immunoblotting quantification was expressed as the fold increase over 9-week-old transgenic male mice. Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote significant difference by age: small letters correspond to normal mice and capital letters to transgenic animals; asterisks indicate significant difference by genotype. ns stands for non-significant. Arrows indicate the quantified bands.

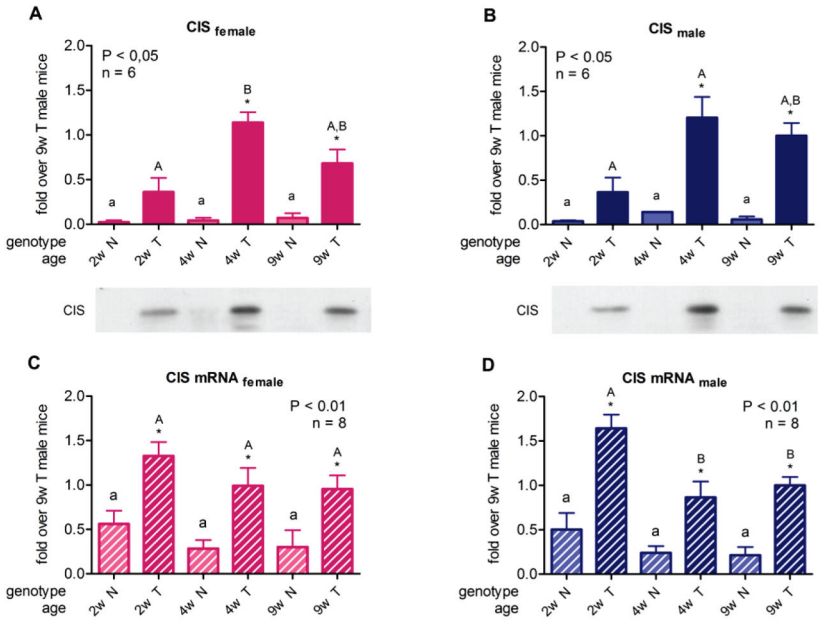

Fig. 4.

CIS expression in liver of growing GH-overexpressing mice and normal littermates. A. CIS protein content in female mice; B. CIS protein content in male mice; C. CIS mRNA expression in female mice; D. CIS mRNA expression in male mice.

To determine protein abundance, CIS required immunoprecipitation to enrich preparation prior to immunoblotting assays; mRNA was assessed by qRT-PCR. Results were expressed as the fold increase over 9-week-old transgenic male mice. Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote significant difference by age; small letters correspond to normal mice and capital letters to transgenic animals. Asterisks indicate significant difference by genotype.

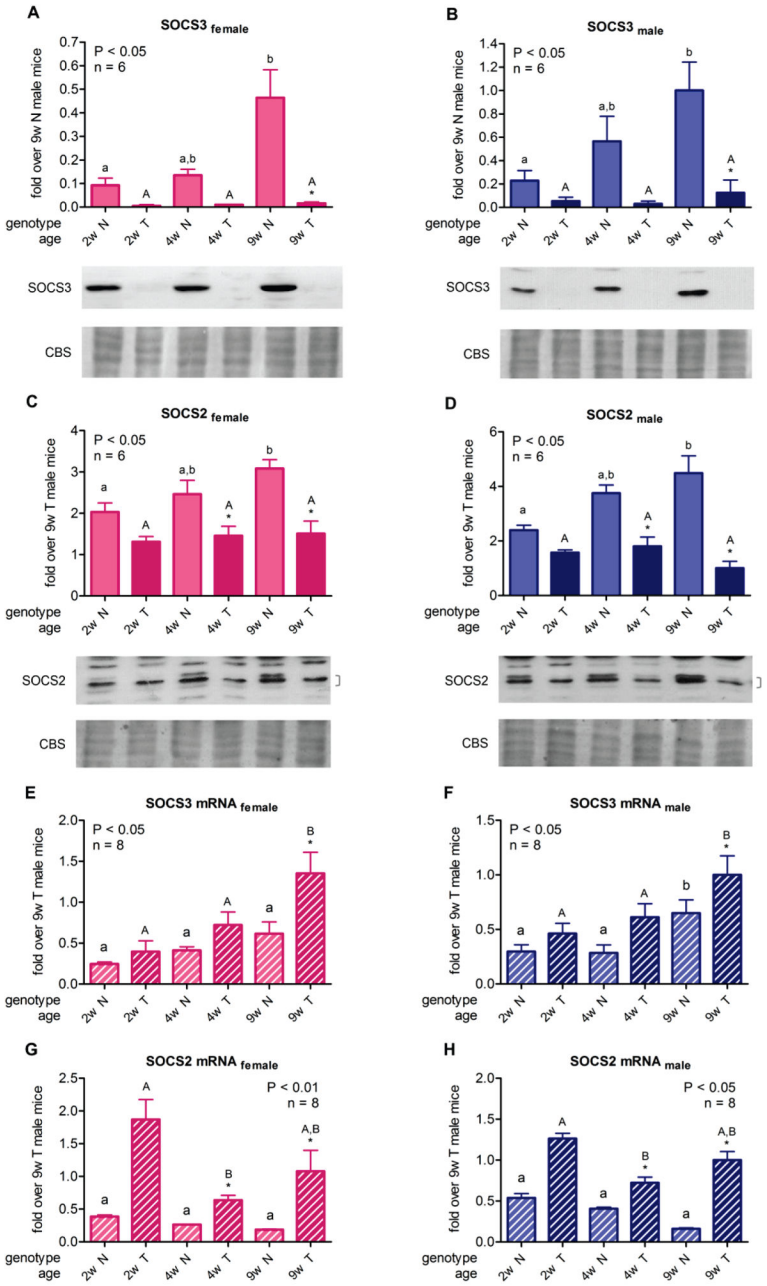

Fig. 5.

SOCS expression in liver of growing GH-overexpressing mice and normal littermates. A. SOCS3 protein content in female mice; B. SOCS3 protein content in male mice; C. SOCS2 protein content in female mice; D. SOCS2 protein content in male mice; E. SOCS3 mRNA expression in female mice; F. SOCS3 mRNA expression in male mice; G. SOCS2 mRNA expression in female mice; H. SOCS2 mRNA expression in male mice.

To determine protein abundance, equal amounts of solubilized liver protein were analyzed by immunoblotting. Coomassie blue staining (CBS) of PVDF membrane is shown. Bands obtained were quantified by scanning densitometry. mRNA was assessed by qRT-PCR from

total RNA extracts. Results were expressed as the fold increase over 9-week-old normal (A,B) or transgenic (C–H) female and male mice. Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote significant difference by age; small letters correspond to normal mice and capital letters to transgenic animals. Asterisks indicate significant difference by genotype. Square brackets indicate the quantified bands.

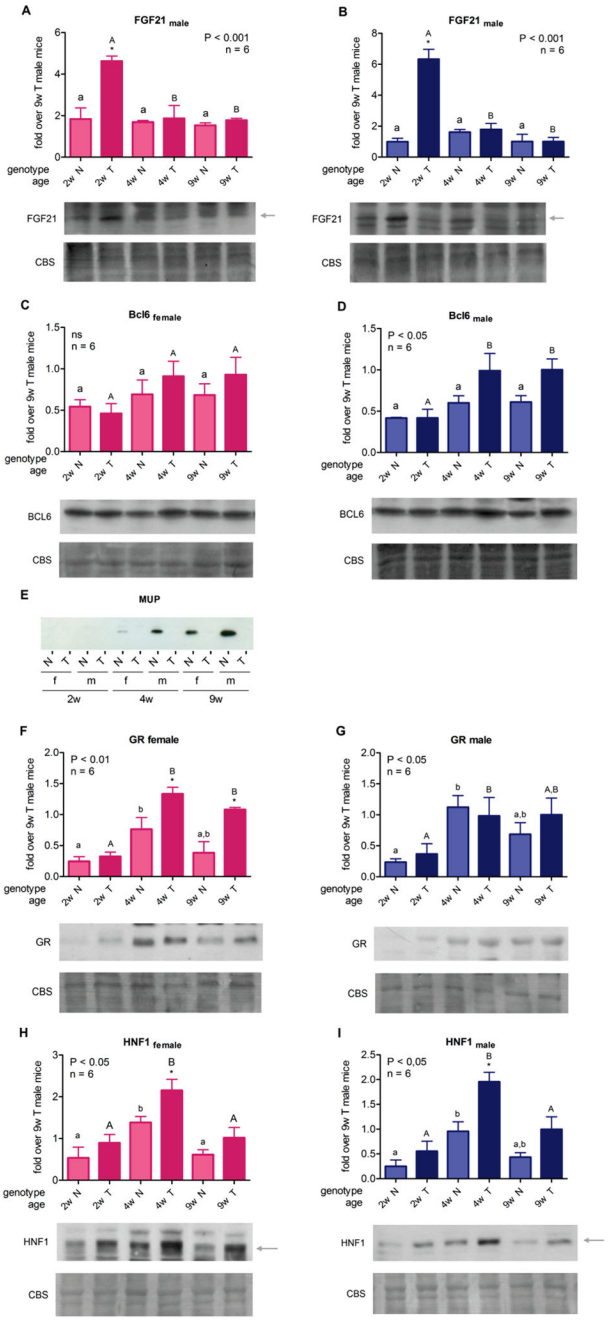

Fig. 6.

STAT5 modulators protein abundance in liver of growing GH-overexpressing mice and normal littermates. A. FGF21 content in female mice; B. FGF21 content in male mice; C. BCL6 content in female mice; D. BCL6 content in male mice. E. Representative immunoblot for MUPs protein content. F. GR content in female mice; G. GR content in male mice; H. HNF1 content in female mice; I. HNF1 content in male mice. Immunoblotting quantification was expressed as the fold increase over 9-week-old transgenic male mice. Data are the mean ± SEM of the indicated n number of samples per group, each one representing a different animal. Different letters denote significant

difference by age; small letters correspond to normal mice and capital letters to transgenic animals. Asterisks indicate significant difference between GH-overexpressing animals and their corresponding non-transgenic age controls. Arrows indicate the quantified bands.

Table 1

Primer sequences used for gene expression assays.

Gene	Primers	Sequence (5′→3′)	Genbank Accesion Number
bGH	forward reverse	GGGCAGATCCTCAAGCAGAC GAAGCAGGAGAGCAGACCG	NM_180996.1
Cis	forward reverse	TGCATAGCCAAGACGTTCTC GTGGGTGCTGTCTCGAACTA	NM_009895.3
Cyclophilin A	forward reverse	GCGTCTCCTTCGAGCTGTT AAGTCACCACCCTGGCAC	NM_008907.1
Ghr	forward reverse	CCAACTCGCCTCTACACC GGGAAAGGACTACACCACCTG	NM_010284.2
Igf1	forward reverse	CTGAGCTGGTGGATGCTCTT CACTCATCCACAATGCCTGT	NM_010512.4
Socs2	forward reverse	TGTGAGTCCCAACCTAGTGC GTAGAAGGGAGGCAGCTGTT	NM_007706.4
Socs3	forward reverse	CTAGGTGAGGAGTGGTGGCT CTGCGAGGTTTCATTAGCTG	NM_007707.3