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Growth hormone STAT5-mediated signaling and its modulation in mice liver during the growth period ☆

Carolina S. Martinez [a], Verónica G. Piazza [a], Laura D. Ratner [b], Marina N. Matos [a], Lorena González [a], Susana B. Rulli [b], Johanna G. Miquet [a], Ana I. Sotelo [a,*]

[a] Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Junín 956, 1113, Buenos Aires, Argentina
[b] Instituto de Biología y Medicina Experimental (CONICET), Vuelta de Obligado 2490, Buenos Aires, Argentina

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ABSTRACT

Postnatal growth exhibits two instances of rapid growth in mice: the first is perinatal and independent of growth hormone (GH), the second is peripuberal and GH-dependent. Signal transducer and activator of transcription 5b (STAT5b) is the main GH-signaling mediator and it is related to IGF1 synthesis and somatic growth. The aim of this work was to assess differential STAT5 sensitivity to GH during the growth period in mouse liver of both sexes. Three representative ages were selected: 1-week-old animals, in the GH-independent phase of growth; 2.5-week-old mice, at the onset of the GH-dependent phase of growth; and 9-week-old young adults. GH-signaling mediators were assessed by immunoblotting, quantitative RT-PCR and immunohistochemistry.
GH-induced STAT5 phosphorylation is low at one-week and maximal at 2.5-weeks of age when compared to young adults, accompanied by higher protein content at the onset of growth. Suppressor CIS and phosphatase PTP1B exhibit high levels in one-week animals, which gradually decline, while SOCS2 and SOCS3 display higher levels at adulthood. Nuclear phosphorylated STAT5 is low in one-week animals while in 2.5-week animals it is similar to 9-week control; expression of SOCS3, an early response GH-target gene, mimics this pattern. STAT5 coactivators glucocorticoid receptor (GR) and hepatic nuclear factor 1 (HNF1) abundance is higher in adulthood. Therefore, GH-induced STAT5 signaling presents age-dependent activity in liver, with its maximum coinciding with the onset of GH-dependent phase of growth, accompanied by an age-dependent variation of modulating factors. This work contributes to elucidate the molecular mechanisms implicated in GH responsiveness during growth.

1. Introduction

Growth hormone (GH) is engaged in the promotion of longitudinal body growth, and participates in multiple cellular processes, including fat and energy metabolism. Its signaling is initiated by ligand binding to a receptor dimer and activation of receptor associated tyrosine-kinase JAK2 [1–3]. The kinase phosphorylates itself and GHR on multiple intracellular tyrosine residues, thus providing docking sites for signaling mediators. Among these, the most important are signal transducers and activators of transcription (STAT), particularly STAT5b. Activated by phosphorylation, these cytoplasmic factors dimerize and translocate to the nucleus. STAT5b is the key mediator of GH-induced IGF1 gene transcription in rodents and of GH-regulated growth [4–7].

The suppressors of cytokine signaling (SOCS)/cytokine-induced suppressor (CIS) proteins modulate cytokine action through a classic negative feedback loop [8–10]. GH promotes the expression of CIS, SOCS1, 2 and 3, with different kinetics [11,12]. These suppressors attenuate GH signaling by two distinct mechanisms: by binding to phosphotyrosine residues on JAK2 or GHR via their SH2 domain, thus impeding other protein interactions, or by driving protein-complexes to proteasome degradation by means of their SOCS-box motif.

Several protein tyrosine phosphatases (PTPs) deactivate GH signaling, including PTP1B and PTPH1 and the cytosolic SH2 domain-containing protein-tyrosine phosphatases (SHP) 1 and 2 [2,9,13–16]. PTP1B was proposed to reduce GH-induced activation of JAK2 therefore limiting the activation of downstream substrates of the kinase [15], although trapping mutant studies have demonstrated that GHR, JAK2 and STAT5b can be substrates of PTP1B themselves [14–16]. PTPH1, which dephosphorylates GHR, JAK2 and STAT5 in vitro [16,17], is the only phosphatase that has been associated with changes in body growth outcome [17]. SHP1 interacts with activated JAK2 [18] and can dephosphorylate STAT5 in the nucleus after GH stimulation [19]. SHP2

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* Corresponding author. Tel.: +54 11 4964 8290, +54 11 4964 8291; fax: +54 11 4962 5457.
E-mail address: aisotelo@qb.ffyb.uba.ar (A.I. Sotelo).

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is a dual player, both promoting and inhibiting JAK/STAT-mediated signaling, acting through its catalytic and SH2 binding domains [20–23].

Glucocorticoid receptor (GR) and hepatocyte nuclear factor 1 α (HNF1α) modulate STAT5 binding to DNA. Liver specific inactivation of GR and HNF1 is associated with reduced somatic growth in mice, affecting STAT5-mediated GH-responsive genes implicated in postnatal growth [24–26]. GR acts as a coactivator of STAT5 [24,25], whereas HNF1α regulates GR expression to control postnatal body growth [26].

Rodents exhibit two phases of rapid growth. The first is the most pronounced, occurs immediately after birth, lasts 2 weeks, and is GH-independent, while the second phase commences around the third week of life, and it is modulated by GH [27–29]. Since GH responsiveness varies during the growth period, and given that STAT5b is the principal GH-mediator in the promotion of body growth, the aim of the present work was to assess GH-STAT5 signaling sensitivity related to the onset of GH action on somatic growth. To this purpose, we have determined STAT5 tyrosine-phosphorylation in liver of mice at different growth stages both basally and after an exogenous GH stimulus, and determined its gene activation capacity, as well as the abundance of regulatory proteins that modulate GH signaling through the JAK2/STAT5 pathway.

2. Materials and methods

2.1. Animals

Swiss female and male mice 7, 17 or 63 days old were used (hereafter referred to as 1-, 2.5- and 9-weeks-old). Additional animals were used to register body weight twice or thrice a week during the first month of life, and once a week afterwards. Animals were housed in a controlled environment with a 12 h light:12 h dark cycle photoperiod (lights on from 06:00 to 18:00 h) and temperature of 20 ± 2 °C. Sentinel animals were tested for all major murine pathogens and the results of the tests were uniformly negative. Animals were given free access to a nutritionally balanced diet and tap water. Housing, handling and experimental procedures followed the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85-2, revised 1985).

2.2. Tissue collection

To assess GH-induced protein phosphorylation and gene expression, mice received 1 μg oGH per gram of body weight by intraperitoneal administration (ovine GH, obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA) between 2 and 4 pm. To evaluate basal conditions, mice were injected with saline for the same period. The animals were sacrificed 7.5 or 30 min after stimulus, livers were removed and stored at −80 °C until solubilization, or were fixed in formalin and included in paraffin.

2.3. Liver solubilization and immunoprecipitation

Tissues were homogenized in 10 volumes of solubilization buffer (1% v/v Triton, 0.1 mol l^{-1} Hepes, 0.1 mol l^{-1} sodium pyrophosphate, 0.1 mol l^{-1} sodium fluoride, 0.01 mol l^{-1} EDTA, 0.01 mol l^{-1} sodium vanadate, 0.002 mol l^{-1} PMSF, and 0.035 trypsin inhibitory units/ml aprotinin, pH 7.4). Homogenates were centrifuged at 100.000 *g* at 4 °C for 50 min to remove insoluble material. Protein concentration of supernatants was determined by the BCA assay (BCA Protein Assay Reagent, Thermo Scientific Pierce, Rockford, IL, USA). An aliquot of solubilized liver was diluted in Laemmli buffer, boiled for 5 min and stored at −20 °C until immunoblotting.

For immunoprecipitation, 4 mg of solubilized liver protein was incubated at 4 °C overnight with 8 μl of anti-CIS antibody (αCIS). After incubation, 15 μl of protein G-Sepharose 50% v/v (Sigma Chemical Co., St. Louis, MO, USA) was added to the mixture. The preparation was further incubated with constant rocking for 2 h at 4 °C and then centrifuged at 1.000 *g* for 30 s at 4 °C. Additional samples were incubated in the absence of immunoprecipitating antibody to assess non-specific binding. The supernatant was discarded, and the precipitate was washed three times with washing buffer (0.05 mol l^{-1} Tris, 0.01 mol l^{-1} vanadate and 1% v/v Triton X-100, pH 7.4). The final pellet was resuspended in 50 μl Laemmli buffer, boiled 5 min, and stored at −20 °C until electrophoresis.

2.4. Nuclear extraction

100 mg of tissue was disrupted in 350 μl hypotonic buffer (0.1% v/v Igepal, 0.01 mol l^{-1} Hepes, 0.0015 mol l^{-1} magnesium chloride, 0.01 mol l^{-1} potassium chloride, 0.01 mol l^{-1} sodium fluoride, 0.0005 mol l^{-1} DTT, 0.0005 mol l^{-1} PMSF, 10 μg/μl leupeptin, 10 μg/μl pepstatin, 0.001 mol l^{-1} sodium vanadate, pH 7.4). Homogenates were sonicated, incubated in ice for 10 min and centrifuged at 10.000 *g* at 4 °C for 2 min. Supernatant was removed and pellet was resuspended in 100 μl hypertonic buffer (0.1% v/v Igepal, 25% v/v glycerol, 0.01 mol l^{-1} Hepes, 0.0015 mol l^{-1} magnesium chloride, 0.420 mol l^{-1} sodium chloride, 0.0002 mol l^{-1} EDTA, 0.01 mol l^{-1} sodium fluoride, 0.0005 mol l^{-1} DTT, 0.0005 mol l^{-1} PMSF, 10 μg/μl leupeptin, 10 μg/μl pepstatin, 0.001 mol l^{-1} sodium vanadate, pH 7.4). Samples were incubated on ice for 20 min and centrifuged at 10.000 *g* at 4 °C for 15 min. Supernatant was recovered and its protein concentration was determined by the BCA assay. An aliquot of sample was diluted in Laemmli buffer, boiled for 5 min and stored at −20 °C until immunoblotting. Nuclear extraction was assessed measuring connective tissue growth factor (CTFG) protein abundance in cytoplasmic and nuclear extracts, indicating a suitable separation when the protein is observed only in the cytoplasmic preparations.

2.5. Immunoblotting

Samples were resolved by SDS-PAGE under reducing conditions and transferred to PVDF membranes (Amersham Hybond-P, GE Healthcare LifeSciences, Pittsburgh, PA, USA). To reduce non-specific antibody binding, membranes were incubated for 1 h at room temperature in T-TBS blocking buffer (0.01 mol l^{-1} Tris–HCl, 0.15 mol l^{-1} NaCl, and 0.02% w/v Tween 20, pH 7.6) containing 3% w/v BSA. The membranes were then incubated overnight at 4 °C with antibodies anti-STAT5 a/b 1:10.000 (αSTAT5; C-17, no. 835), anti-CIS 1:200 (αCIS; N-19, no. 1529), anti-SOCS2 1:600 (αSOCS2; H-74, no. 9022), anti-SOCS3 1:300 (αSOCS3; H-103, no. 9023), anti-HNF1 1:300 (αHNF-1; H-205, no. 8986), anti-GR 1:300 (αGR; M-20, no. 1004), anti-CTGF 1:500 (αCTGF, no. 14939) 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. catalog 05-495), anti-PTP1B 1:2.500 (αPTP1B, no. 07-088) purchased from Millipore (Billerica, MA, USA); anti-actin 1:1.000 (αβ-actina, no. 2066) obtained from Sigma Aldrich; anti-tubulin 1:10.000 (αβ-tubulina, no. 6046) from Abcam (Cambridge, MS, USA); or anti-GHR antiserum (αGHR) 1:1000 generously provided by Dr. S.J. Frank [30]. 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).

For reblotting, membranes were stripped by incubation for 30 min at 50 °C in buffer containing 0.0625 mol l^{-1} Tris–HCl, 2% w/v SDS, 0.1 mol l^{-1} mercaptoethanol, pH 6.7. Blots were washed, reblotted, and immunolabeled as described above.

2.6. Immunohistochemistry

Freshly dissected livers were fixed in 10% formalin and embedded in paraffin. Liver sections (5 μm thickness) were transferred onto glass slides, deparaffinized, rehydrated and subjected to antigenic recuperation with sodium citrate (pH 6.0) at 98 °C for 30 min. Endogenous peroxidase activity was blocked incubating slides on PBS containing 3% v/v hydrogen peroxide (H_2O_2) for 30 min. Non-specific proteins were blocked using PBS containing 1% w/v BSA for 1 h and then normal horse serum for 2 h (R.T.U. vectastain kit, Vector Laboratories, Burlingame, CA, USA). Incubation with antibodies anti-HNF1 1:50 (αHNF-1; H-205, no. 8986), anti-GR 1:50 (αGR; M-20, no. 1004) purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA) was performed overnight at 4 °C; incubation with biotin-labeled secondary antibodies followed by incubation with streptavidin-horse radish peroxidase complex, were each performed for 30 min at room temperature. Tissues were incubated with normal horse serum instead of the primary antibody as negative control. The antigen-antibody binding was visualized with the DAB chromogen (Peroxidase substrate kit, DAB sk-4100, Vector Laboratories), counterstaining was with hematoxylin. Sections were dehydrated through 96% ethanol for 1 min and absolute ethanol for 3 min, and cleared in xylene for 5 min. Slides were mounted with balsam. Quantification of GR and HNF1 was estimated as percentage of positive (brown stained) nuclei in 500 cells approximately. The counting was performed under light microscopy.

2.7. Real-time reverse transcriptase PCR

Total hepatic RNA was extracted using the phenol chloroform method [31]. Two micrograms of RNA were treated with DNAseI (Invitrogen) and reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's instructions. To validate successful DNase I treatment, the reverse transcriptase was omitted in control reactions. The absence of PCR-amplified DNA fragments in these samples indicated the isolation of RNA free of genomic DNA. For quantitative real-time RT-PCR primer sets were designed for the specific amplification of *Socs3* and *Cyclophilin* as a housekeeping gene. Sense and antisense oligonucleotide primers were designed by the use of the software Oligo Calc: Oligonucleotide Properties Calculator [32]. Oligonucleotides were obtained from Invitrogen. Each sample was assayed in duplicate using 4 pmol of each primer, 1× SYBR Green Master Mix (Applied Biosystems) and 0.1–1 μl of cDNA in a total volume of 13 μl. Amplification was carried out in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The results were validated on the basis of the quality of dissociation curves generated at the end of the PCR runs by ramping the temperature of the samples from 60 to 95 °C, while continuously collecting fluorescence data. Each sample was analyzed in duplicate. Relative gene expression levels were calculated according to the comparative cycle threshold (Ct) method. Normalized target gene expression relative to cyclophilin A, used as housekeeping gene, was obtained by calculating the difference in Ct values, the relative change in target transcripts being computed as $2^{-\Delta Ct}$. To validate the comparative Ct method of relative quantification, the efficiencies of each target and housekeeping gene amplification (endogenous cyclophilin A) were measured and shown to be approximately equal.

2.8. Statistical analysis

Results are presented as means ± standard error (SEM) of the *n* number of samples (different individuals) indicated. Experiments were performed analyzing all groups of animals in parallel. The number of separate experiments performed is indicated in each figure. Statistical analysis was performed by two way ANOVA followed by the Bonferroni test using the GraphPad Prism statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Data were considered significantly different if $P<0.05$.

3. Results

3.1. Age selection

Growth in rodents exhibits two stages of rapid growth, the first is perinatal and the second peripuberal [29]. Fig. 1A shows the variation of body weight with age for male and female mice, while in Supplementary Fig. 1A data is displayed as body weight gain (%) vs. age. Body weight increment is higher perinatally; after a pause in body weight gain (around week 2) the second phase of rapid growth begins; at this stage, growth rate is maximal between weeks 2.5 and 4 (Suppl. Fig. 1A).

To assess GH-induced STAT5 response in liver, mice between one and 4-weeks of age were examined; 9-week young adults were used as control. In order to evaluate several ages simultaneously, only female mice were included in this initial study. Tyrosine-phosphorylation of STAT5 proteins was evaluated by immunoblotting of liver extracts with anti-phospho-STAT5a/b antibody. After a 7.5 min GH-stimulus, maximal phosphorylation levels were achieved at 2 and 3 weeks of age (Suppl. Fig. 1B). Therefore, in order to evaluate GH response for both sexes in parallel at different growth periods, three representative age points were chosen: lactating one-week (1w) mice, pre-weaning (2.5w) mice, and young adults (9w). These ages were selected as: perinatal or in the GH-independent phase growth; at the beginning of the GH-dependent growth, when maximal GH-induced STAT5 phosphorylation was observed in female mice (Suppl. Fig. 1B), and the young adult, when growth rate has decelerated. These age points are indicated by arrows in Fig. 1A. Female and male mice were assayed in parallel since GH secretion is sexually dimorphic and this has been associated with sex-specific gene expression and the major corporal growth of male mice after weaning [33,34].

3.2. GHR abundance

Hepatic GHR content increased according to age; one-week-old mice exhibited approx. 25% of young adult values while 2.5-week mice presented approx. 70% of respective control (Fig. 1B), similar to that described for the rat [35–37].

3.3. STAT5 activation

To assess GH-induced STAT5 phosphorylation, animals received an exogenous GH-stimulus. Nine-week-old mice were used as age control for each sex group, and 9-week female mice were referred to as 100%. Results displayed a similar pattern for each sex. At every age point evaluated, higher STAT5 phosphorylation was achieved upon GH stimulus when compared to basal response. At one week of age, GH-induced STAT5 phosphorylation values were half of those of young adults, while at 2.5 weeks they were 1.5-fold of 9-week mice (Fig. 1C). No sex difference was found at any age point.

When STAT5a/b protein content was assayed, one-week-old mice exhibited the same values as 9-week control, whereas at 2.5-weeks of age, a two-fold increase over the values determined for 9-week mice was found for both sexes (Fig. 1D). For quantification purposes, STAT5 protein content was determined in non-stimulated mice tissues only, to avoid distortion due to differential migration of phosphorylated species [38–40].

When phospho-STAT5 values were normalized to STAT5 protein content (Fig. 1E), the pSTAT5/STAT5 ratio for 1-week mice was around half of 9-week control mice. As STAT5 protein content is also increased at 2.5-weeks of age, the pSTAT5/STAT5 ratio was also lower at this age than for the 9-week reference for both sexes, although the difference did not achieve statistical significance. Changes in the phosphorylation status of STAT5 do not seem to be solely due

to variation in STAT5 protein abundance, since at 1-week of age, phosphorylation levels were lowest, even when STAT5 protein content was similar to that of young adult mice.

When basal and GH-stimulated samples are assayed together, the high intensity of GH-stimulated phosphorylation bands blunts the basal phosphorylation signal in non-stimulated animals, rendering it almost undetectable (Fig. 1C). To better assess basal STAT5-phosphorylation, which is elicited by endogenous stimuli, samples from non-stimulated animals were run separately, to avoid interference by the intense signal of stimulated ones [41]. The age variation found, although in coincidence with that induced by stimulation, did not reach statistical significance (Fig. 1F). Moreover, reported differences between sexes could not be seen. To address this point, samples were rearranged in sets by age in order to reduce the number of groups under study with the concomitant

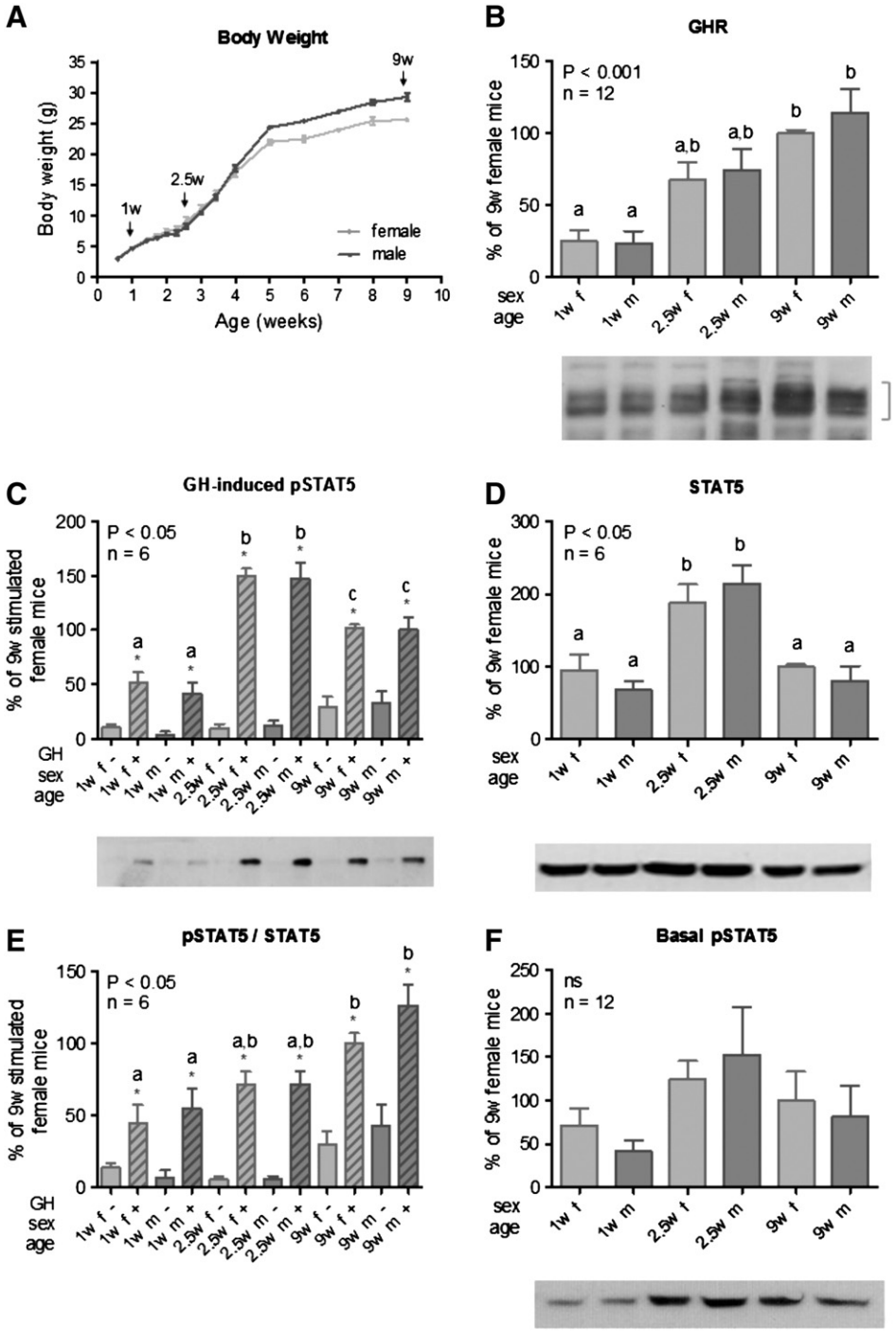

Fig. 1. Hepatic GHR/STAT5 signaling in growing mice. A. Body weight vs. age. Arrows indicate ages selected for the experiments shown in this figure and Figs. 2–5. n = 6 for male and female mice. B. GHR content; C. GH-induced tyrosine phosphorylation of STAT5; D. STAT5 protein abundance; E. GH-induced tyrosine phosphorylation/protein content ratio for STAT5; F. Endogenous (basal) tyrosine phosphorylation of STAT5. Mice of indicated ages were injected *ip* with oGH 1 μg/g of body weight (+; filled bars) or saline (−; open bars), killed after 7.5 min and livers removed. Equal amounts of solubilized liver protein were analyzed by immunoblotting. Protein content and phosphorylation were quantified by scanning densitometry and expressed as a percentage of the mean value measured for 9-week old female mice. Data are the mean ± SEM of the indicated *n* number of samples per group, each one representing a different animal. Groups were analyzed in parallel, six separate experiments were performed. Different letters denote significant differences, whereas results with the same letter are not statistically different from each other; asterisks indicate significant difference between GH-stimulated animals and their corresponding basal pair. In Fig. 1B, bracket indicates the quantified band.

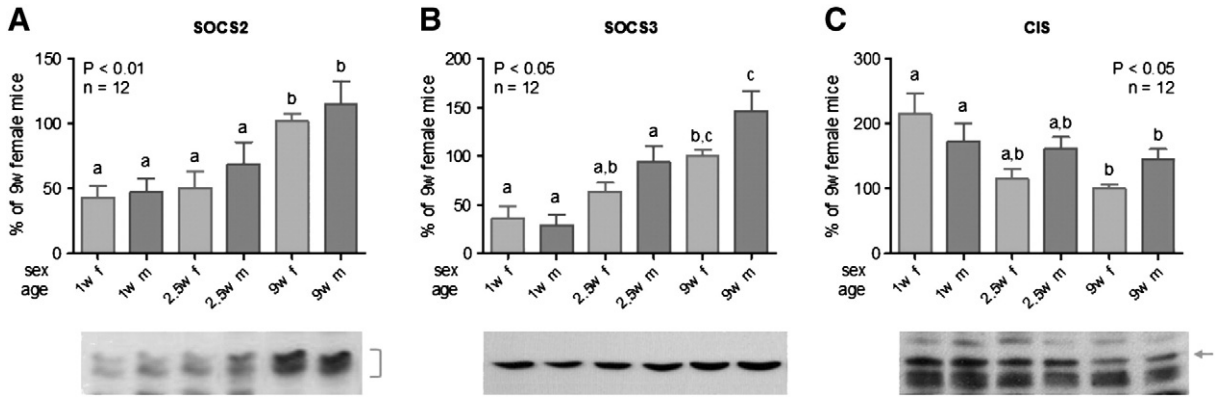

Fig. 2. SOCS protein abundance in liver of growing mice. A. SOCS2 content, B. SOCS3 content, C. CIS content. To determine protein abundance, equal amounts of solubilized liver protein were analyzed by immunoblotting; CIS required prior immunoprecipitation to enrich preparation. Protein content was quantified by scanning densitometry and expressed as a percentage of the mean value measured for 9-week old mice. Data are the mean ± SEM of the indicated *n* number of samples per group, each one representing a different animal. Six separate experiments were performed. Different letters denote significant differences, whereas results with the same letter are not statistically different from each other; bracket (2A) and arrow (2C) indicate the quantified band.

increment of the number of samples (n) determined in the same experiment. Under these conditions, statistical significance was found only for 9-week animals, both basally (Supp. Fig. 2A-C) or after the exogenous GH-stimulus (Supp. Fig. 2D–F), with 9-week old males achieving higher response.

3.4. SOCS protein content

SOCS2, SOCS3 and CIS are the GH-induced suppressors of cytokine signaling expressed in the liver. Thus, the hepatic abundance of these suppressors was determined by immunoblotting in mouse liver homogenates. SOCS2 presented lower levels at early ages compared to young adults; 1-week mice presented approximately 45% of young adult values while 2.5-week mice represented 55% of the same reference group (Fig. 2A). Hepatic SOCS3 content presented a similar tendency; 1-week-old mice values were 30–35% of young adults while for 2.5-week animals, values were approximately 60% of their respective 9-week reference (Fig. 2B), suggesting that these SOCS proteins modulate GH action mostly in the adult.

When CIS abundance was analyzed, higher levels were found at early ages. One-week animals exhibited the greatest values, 2.5-fold higher than 9-week female control. For 2.5-week animals, values were approx. 1.4-fold over their respective 9-week controls, although this difference did not reach statistical significance (Fig. 2C). High CIS levels could be preventing STAT5 activation in one-week animals.

3.5. Phosphatase protein content

GH-signaling is also limited by dephosphorylation of activated mediators by specific phosphatases. PTP1B, SHP1 and SHP2 are recruited to activated GHR complex to extinguish GH signal. Hepatic content of these enzymes was assayed by immunoblotting of mouse liver homogenates. PTP1B was present at maximum levels at 1-week of age, which declined thereafter. For 1-week mice, values were approximately 5-fold higher than those for young adults, while for 2.5-week old mice values were almost 2-fold higher than reference (Fig. 3A). SHP1 and SHP2 hepatic content did not significantly differ with sex or age (Fig. 3B and C).

3.6. STAT5 nuclear coactivators

Glucocorticoid receptor (GR) and hepatic nuclear factor 1 (HNF1) do not belong to the GH signaling cascade, but participate modulating STAT5 activity. Since STAT5 exhibits higher content with conserved activation capacity at 2.5-weeks, the following step was to determine if these higher levels were also accompanied by high levels of coactivators during the growth period. The hepatic abundance of GR and HNF1 in liver was assessed in an enriched nuclear fraction by immunoblotting of nuclear extracts and they were also determined by immunohistochemistry (IHC) of tissue sections (Fig. 4).

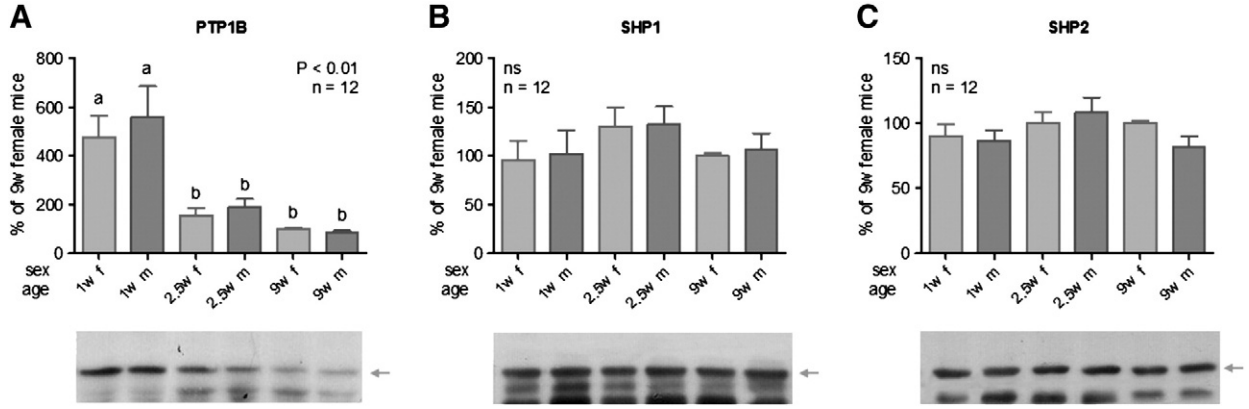

Fig. 3. Phosphatase protein abundance in liver of growing mice. A. PTP1B content, B. SHP1 content, C. SHP2 content. To determine protein abundance, equal amounts of solubilized liver protein were analyzed by immunoblotting. Data are the mean ± SEM of the indicated *n* number of samples per group, each one representing a different animal; six 6 separate experiments were performed. Different letters denote significant difference, whereas results with the same letter are not statistically different from each other; arrows indicate the quantified band.

GR abundance increased with age, 1-week mice presented approx. 15% of young adult values, while 2.5-week mice presented about 50%, both for female and male mice when assessed by immunoblotting (Fig. 4A). For HNF1, the same profile was observed. Immunoblotting analysis revealed that one-week mice presented around 15% of young adult values and 2.5-weeks mice presented 60% of their respective controls, both for female and male mice, although the differences between the two last age-groups did not reach statistical significance (Fig. 4B). When assayed by IHC, positive nuclei were counted and the same pattern was observed for both coactivators, albeit at different proportions (Fig. 4C and D). Therefore, maximal STAT5 activation found at 2.5-weeks is not accompanied by higher levels of GR and HNF1.

3.7. STAT5 nuclear translocation and gene activation

Nuclear translocation capacity was assessed by immunoblotting of nuclear extracts and by immunohistochemistry of liver sections after a 30 min GH-stimulus in order to determine if the high STAT5 phosphorylation induced by exogenous GH would be functional. For GH-induced STAT5 phosphorylation one-week animals presented a third of 9-week reference values, and 2.5-week animals displayed similar values as those of young adults (Fig. 5A). Basal STAT5-phosphorylation was not detected in any of the samples when run in parallel with stimulated ones. Samples had to be run separately, optimizing conditions, to achieve basal detection (results not shown). In this instance, individual values oscillated between high or undetectable STAT5 phosphorylation levels, as previously demonstrated for the mice liver [42]. Phosphorylation of STAT5 could not be determined by IHC.

Although STAT5 protein content in the nucleus is expected to reflect nuclear translocation of the activated factor [39,43], we found high STAT5 content in non-stimulated samples of the indicated ages both by immunoblotting (Fig. 5A) and by IHC (Fig. 5B), even when tyrosine phosphorylation was not straightforwardly observed (Fig. 5A). Significant differences between basal and GH-stimulated nuclear STAT5 content were found for 2.5-week and for 9-week male and

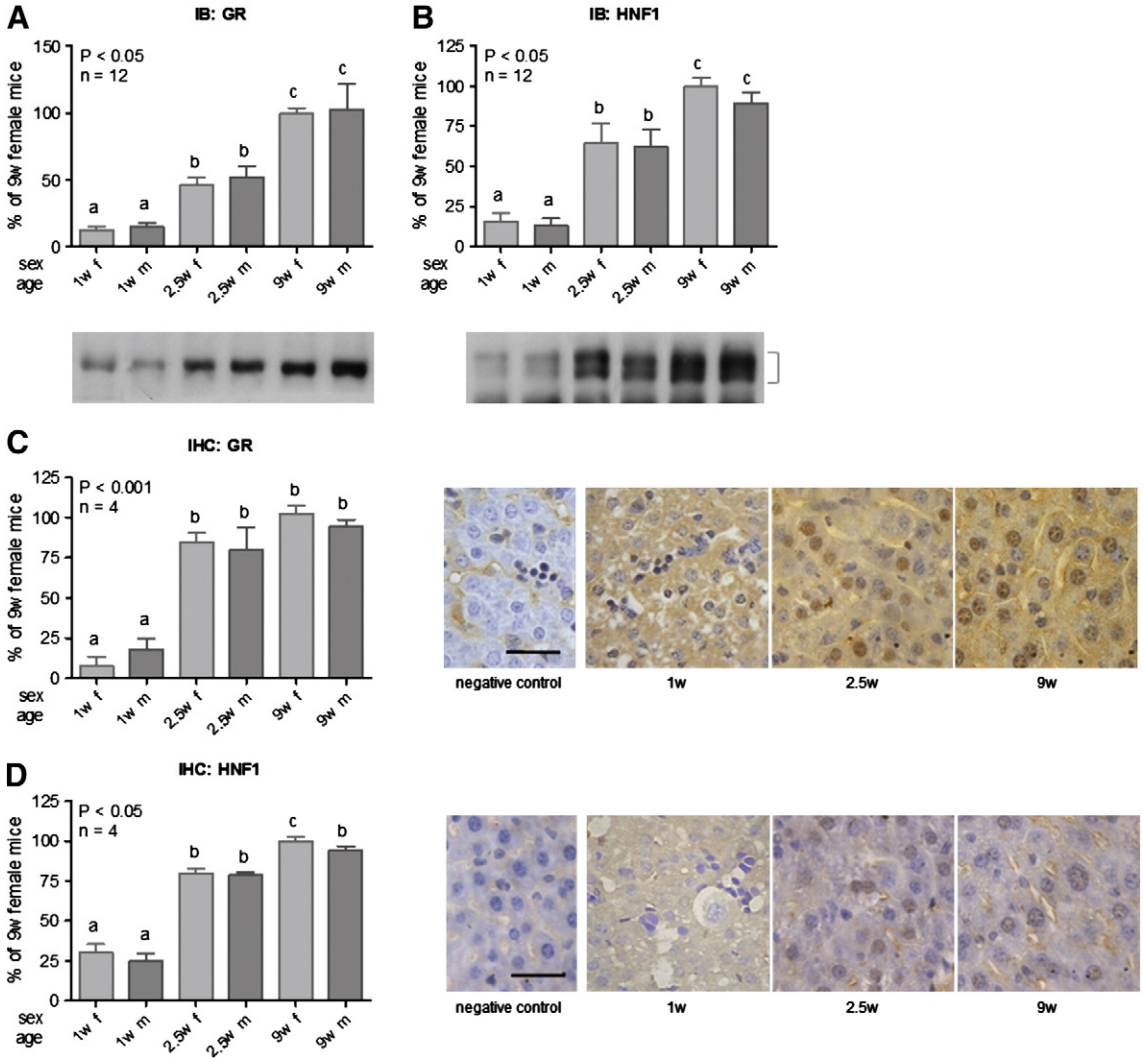

Fig. 4. Nuclear STAT5 coactivator abundance in liver of growing mice. A. GR content by immunoblotting, B. HNF1 content by immunoblotting, C. GR abundance by immunohistochemistry, D. HNF1 abundance by immunohistochemistry. Nuclear content of GR and HNF1 was determined by immunoblotting (A and B) for both sexes. Mice liver was cell-fractionated and the nuclear enriched fraction was analyzed. Protein abundance was quantified by scanning densitometry and expressed as a percentage of the mean value measured for GH-stimulated 9-week old female mice. Data are the mean ± SEM of the indicated *n* number of samples per group, each one representing a different animal. Six separate experiments were performed. Different letters denote significant differences, whereas results with the same letter are not statistically different from each other. Immunohistochemistry (C and D) on liver slides was performed to assess GR and HNF1. Scale bar = 50 μm. Only microphotographs for male mice are shown. For quantification, GR and HNF1 content was calculated as percentage of positive (brown stained) nuclei in 500 cells approximately for both sexes. Results are expressed as a percentage of the mean value measured for 9-week old female 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 differences, whereas results with the same letter are not statistically different from each other.

female mice, but not for one-week animals (Fig. 5B). An age-variation pattern of STAT5 nuclear content as that found in whole tissue extracts could not be established. As a control of cytosolic contamination of nuclear extracts, connective tissue growth factor (CTFG) –a cytoplasmic protein [44]– was measured both in nuclear and cytosolic fraction (Fig. 5A). CTFG levels were very low in the nuclear fraction, thus excluding cytosolic contamination of nuclear fraction to account for high STAT5 nuclear content.

In order to assess the activity of the phosphorylated transcription factor, we determined induction of STAT5-mediated gene SOCS3 after 30 min of GH stimulus. SOCS3 is an early response gene activated by GH to terminate its own signal [11,12]. We found exogenous GH-induced gene activation above basal levels for every age studied and for both sexes, although this difference was non-significant for one-week animals (Fig. 5C).

3.8. Loading control

It is worth mentioning that actin could not be used as loading control for immunoblotting, since this protein exhibited age-dependent variation in hepatic protein abundance (Suppl. Fig. 3A). Coomassie blue staining was used to evaluate homogeneity of sample preparation for the different sets of samples, and Ponceau staining was used to assess goodness of electrotransference [45] (Suppl. Fig. 3B). Tubulin content was determined to assess uniformity of loading (Suppl. Fig. 3C).

4. Discussion

Postnatal growth in mice is described as triphasic, exhibiting two instances of rapid growth [27–29]. The first and most rapid one occurs immediately after birth and is independent of GH. After a short pause, the GH-dependent phase of rapid growth, known as the growth spurt, takes place. Mice then continue to grow at a slower rate, before reaching the growth plateau (adult size). Coinciding with the growth spurt, circulating GH levels increase during the prepubertal period in the rat, and decrease afterwards in the sexually mature animal [46]. The second period of rapid growth is preceded by a rise in serum IGF1 concentration and hepatic IGF1 mRNA levels [29,47,48]. The precise timing of the endocrine transition to GH-dependent growth is not well defined, but the onset of GH activity on growth represents the conversion from the autocrine/paracrine control of growth to the central regulation [49].

Lack of GH influence on somatic growth during fetal and postnatal periods has been attributed to insufficient GH receptor number or to immature GHR forms. GHR mRNA abundance has been reported to be barely detectable in fetal and early postnatal rat liver [36], while other authors have described both GH and GHR in the fetus [50,51]. GH-induced STAT5 phosphorylation in liver of fetal rats has been demonstrated in vivo [52]. In the current work, GH signaling during the growth period was evaluated in mouse liver and GH-induced STAT5 phosphorylation was found at every age point studied. One-week-old mice respond to GH, reflecting that GH-independent growth is not

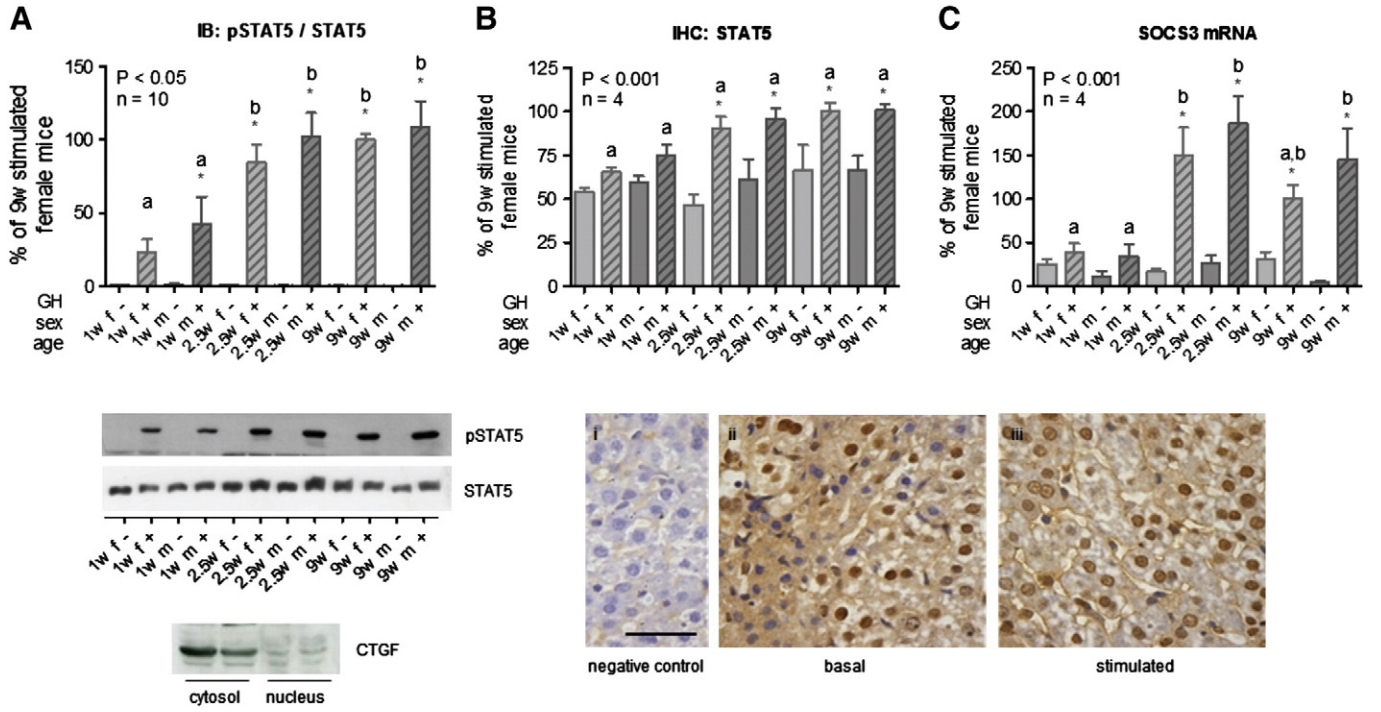

Fig. 5. STAT5 nuclear translocation and gene activation in liver of growing mice. A. Nuclear Phospho-STAT5 content. pSTAT5/STAT5 ratio, assessed by quantification of immunoblotting (IB). Immunoblotting of phospho-STAT5 in nuclear extracts after 30-min GH stimulus and STAT5 nuclear content. CTGF control of nuclear extraction. CTGF is a cytosolic protein, scarcely detected in the nuclear fraction. B. STAT5 nuclear content, assessed by immunohistochemistry (IHC). Panels show nuclear stain for control (i, without first antibody), basal (ii, saline-treated animals) and 30-min GH-stimulated (iii) samples. Scale bar = 50 μm. C. SOCS3 gene induction after 30-min GH stimulus, assessed by quantitative RT-PCR. Mice of indicated ages were injected *ip* with oGH 1 μg/g of body weight (+; filled bars) or saline (−; open bars), killed after 30 min and livers removed. Data are the mean ± SEM of the indicated *n* number of samples per group, each one representing a different animal. Same letter indicates that results are not statistically different from each other, whereas asterisks indicate significant difference between GH-stimulated animals and their corresponding basal pair. Nuclear STAT5 phosphorylation and content were determined by immunoblotting (A) for both sexes in a nuclear enriched fraction. Protein abundance was quantified by scanning densitometry and expressed as a percentage of the mean value measured for GH-stimulated 9-week old female mice. Ten separate experiments were performed. Immunohistochemistry (B) on liver slides was performed to assess STAT5 nuclear location in the tissue. For quantification, STAT5 content was calculated as percentage of positive (brown stained) nuclei in 500 cells approximately for both sexes. Results are expressed as a percentage of the mean value measured for 9-week old female mice. Only microphotographs for 2.5-week-old female mice are shown. For qRT-PCR (C), samples were analyzed in duplicate. All groups were run in parallel in two separate experiments. Ciclophylin A was used as house-keeping gene.

due to GH insensitivity in the liver, although this response is only half of that of adult mice, which could be related to the low hepatic GHR content found in 1-week mice and to high levels of the negative modulators CIS and PTP1B.

Highest GH-induced STAT5 phosphorylation was observed in 2.5w-mice, coinciding with the onset of growth spurt in these animals. At this age, STAT5 protein content also increases therefore contributing to the augmented response found. We could not detect statistically significant differences in basal STAT5 activation at any age or sex, not even in the adult reference, contrary to that observed for the rat in which both age and sex differences were found [53]. It was necessary to rearrange sets by age to achieve sex differences (Supp. Fig. 2), which were found for the young adult only, both in basal and GH-induced STAT5 phosphorylation. This is not surprising, since younger animals are prepuberal, therefore sex-related changes may have not been yet established.

SOCS proteins impair JAK2/STAT-mediated signaling. Therefore, the abundance of the SOCS proteins that modulate GH action was assessed for the period in study. CIS has been regarded as an important player in the down-regulation of GH-signaling in vivo [9,10,38,54,55]. We have previously described a direct relationship between circulating GH levels and hepatic CIS expression in mice overexpressing or lacking GH, which was inversely related to GH-induced STAT5 phosphorylation [56–58]. However, in the growing mice, CIS hepatic expression does not coincide with the reported maximum of circulating GH occurring during the growth spurt. This suggests that the ontogenic expression of CIS contributes to the partial GH-insensitivity found at young ages.

SOCS2 is the only SOCS protein that was related to the growth outcome [9,59,60]. Recently SOCS2 was shown to bind SOCS1 and SOCS3, and target them to proteosomal degradation, thus regulating their turnover [8,9,61,62]. In growing mice, SOCS2 and SOCS3 content present an age-dependent pattern reaching the maximum at adulthood, suggesting SOCS2 and SOCS3 would modulate GH activity preferentially in adults.

Deactivation of GH-induced kinase-phosphorylated signaling mediators involves the action of several phosphatases. Phospho-STATs are mostly recycled through tyrosine-dephosphorylation, while receptor and kinase are principally degraded [63]. Therefore, the abundance of protein tyrosine phosphatases that modulate GH action was assessed during the growth period in mice liver. SHP1 and SHP2 present no age difference, while PTP1B content is elevated in one-week mice and decreases thereafter. Our results suggest that PTP1B could be participating in the rapid dephosphorylation of activated substrates at early ages, thus impeding GH signaling, in agreement with recent observations in which STAT5 phosphorylation was found augmented in PTP1B -/- 3 day old mice [64], and that rapid STAT5 dephosphorylation was described when PTP1B was overexpressed [14]. Mice lacking functional SHP1, SHP2 or PTP1B do not present enhanced growth [65–67], while mice lacking the PTPH1 catalytic domain exhibit a modest increment in body weight, especially in male mice. This is particularly evident from the third week of age, reflecting the relevance of this phosphatase in controlling GH signaling and body growth outcome [17]. Unfortunately, we were not able to determine the hepatic content of this phosphatase, probably because its abundance in the liver is low [17].

Even when no statistically significant differences could be detected in basal STAT5 phosphorylation, the higher STAT5 protein content found at 2.5-weeks of age suggested the activation of this transcription factor would be physiologically relevant for that age point. However, GH-induced proteins like GHR or SOCS/CIS are not up-regulated at that age. Therefore, we sought to elucidate if the higher phosphorylation levels induced by exogenous GH stimulus could lead to higher gene induction. We determined SOCS3 mRNA levels after a 30 min GH-stimulus, which exhibited the expected profile in concordance with phospho-STAT5 nuclear content. Even when phospho-STAT5 is dephosphorylated in the nucleus prior to be recycled to the cytoplasm, the high STAT5 nuclear content we found both by immunoblotting and IHC in non-stimulated samples is too high to be merely attributed to the recycling of the factor; moreover, STAT5 has been reported to undergo intense nuclear translocation even if it is not phosphorylated [68,69]. Cytosolic contamination of the nuclear extract was excluded by CTGF control; moreover, nuclear STAT5 localization in basal samples was also confirmed by IHC.

STAT5s require cooperation or interaction with other proteins to induce gene transcription, since they are weak activators [63]. Glucocorticoid receptor (GR) has an essential role in the control of body growth, serving as a coactivator for GH-induced STAT5-dependent transcription in liver [24,25]. Hepatocyte nuclear factor 1α (HNF1α) is a transcription factor required for postnatal growth. Global HNF1 deletion results in reduced somatic growth, which can be restored by reexpression of the protein in the liver [26,70]. HNF1 has been shown to directly regulate the transcriptional activity of the GR, and thus the expression of GR-mediated GH responsive genes [26]. In liver of growing mice, GR exhibits an age-dependent pattern, with its major level in adults and, interestingly, HNF1 presents the same profile as GR in this tissue, probably reflecting HNF1 regulation over GR transcription. Neither of these proteins has been shown to be regulated by GH [71].

Although IGF1 is considered the principal mediator of the somatotropic function of GH, the involvement of liver derived IGF1 in somatic growth has been questioned (reviewed by [72]). In the liver, IGF1 production requires GH-induced gene transcription activation, being STAT5 the principal mediator involved. Even when systemic STAT5b deletion leads to a reduction of body size in rodents [6], STAT5b combined muscular and hepatic deletion had no further impact on body weight than the reduced body size observed after tissue-specific muscle disruption of the transcription factor [73]. On the other hand, hepatocyte deletion of STAT5a/b led to impaired body growth [24]. Conversely, liver specific growth hormone receptor ablation was shown to have no incidence on total body and bone linear growth [74]. Work involving GR and HNF1 demonstrate not only that liver is involved in somatic growth [25], but also that STAT5 activity in hepatocytes is required for normal postnatal body growth [24].

In summary, GH is known to be expressed at low levels after birth and to rise during puberty. This period is accompanied by a major change in body size, known as the growth spurt. Higher phosphorylation capacity of hepatic STAT5 coincides with the onset of the GH-dependent phase of growth. This result can be interpreted in light of the ontogeny of signaling molecules, as GHR and STAT5, and modulators, like CIS and PTP1B, since a combination of rise and fall in the levels of these mediators takes place during the growth spurt.

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Conflict of interests

None declared.

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