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Growth hormone administration patterns differently regulate epidermal growth factor signaling

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Abstract

Current growth hormone (GH) administration protocols require frequent subcutaneous injections, resulting in suboptimal compliance. Therefore, there is interest in developing delivery systems for sustained release of the hormone. However, GH has different actions depending on its continuous or pulsatile plasma concentration pattern. GH levels and circulating concentration patterns would be involved in the regulation of epidermal growth factor receptor (EGFR) expression in liver. Aberrant expression of this receptor and/or its hyperactivation have been associated with pathogenesis of different types of carcinoma. Considering that one of the adverse effects associated with GH overexpression and chronic use of GH is the increased incidence of malignancies, the aim of this study was to analyze the effects of GH plasma concentration patterns on EGFR expression and signaling in mice liver. For this purpose, GH was administered by subcutaneous daily injections to produce an intermittent plasma pattern or by osmotic pumps to provoke a continuous GH concentration. Results showed that intermittent injections of GH induced an up-regulation of liver EGFR content, augmented the response to EGF and the induction of proteins involved in cell proliferation promotion in female mice. On the contrary, continuous GH delivery in male mice was associated with diminished EGFR liver content and decreased EGF-induced signaling and expression of early genes. Results suggest that sustained delivery systems that allow continuous GH plasma patterns would be beneficial in terms of treatment safety referred to its actions on EGFR signaling and its promitogenic activity.

Introduction

Growth hormone (GH) is a pituitary hormone that stimulates longitudinal bone growth; it induces diverse effects on cell growth and differentiation, and on the metabolism of proteins, lipids and carbohydrates (Herrington & Carter-Su 2001). The use of growth hormone in the endocrinological practice and for the treatment of various clinical conditions is expanding. The first use of human GH (hGH) was as replacement therapy in children with GH deficiency (GHD). However, further indications have been gradually approved or proposed since the development of recombinant human GH (rhGH) (Laron 2011, Kemp & Frindik 2011). Current protocols for growth hormone treatment imply its subcutaneous or intramuscular injection once daily or three times a week. The main disadvantages of these administration protocols are the short plasma half life of the hormone and its renal toxicity. Moreover, injection results in poor patient compliance, high dose, non-specific toxicity and increased cost (Kwak *et al.* 2009, Park *et al.* 2013). Thus, development of sustained-release rhGH formulations could improve patient quality of life and decrease secondary effects (Laron 2011). To date, a once-weekly sustained release GH preparation has shown to be effective for the treatment of several clinical conditions (Biller *et al.* 2012, Péter *et al.* 2012) and sustained delivery systems that last longer are being investigated (Wei *et al.* 2012, Park *et al.* 2013). However, GH shows differential effects depending on its plasma concentration pattern in many species including mice, rats and humans (Jansson *et al.* 1985, Jaffe *et al.* 2002, Waxman & O'Connor C 2006). Therefore, the efficacy and toxicity of pharmaceutical systems that allow prolonged release of the hormone, which would produce near continuous GH circulating levels, should be assessed and compared with the effects of the treatments that involve intermittent injections and mimic a pulsatile concentration pattern.

The differential effects of intermittent and sustained plasma GH patterns have been studied in humans and animals mostly focusing on the consequences on longitudinal growth and metabolism; however, the differential effects of plasma GH patterns on the activation of signaling pathways involved in cell proliferation and survival have not been analyzed. A relevant side effect associated

with chronic use of GH and its overexpression is the increased risk of malignancies (Jenkins 2006, Siobhan & Shereen 2008, Laron 2011). Previous studies suggested that growth hormone concentration patterns regulate epidermal growth factor receptor (EGFR) expression in rodent liver. The EGFR, also known as ErbB-1, is a plasma membrane glycoprotein which belongs to the ErbB family of receptor tyrosine kinases (RTKs) (Burgess 2008). Upon ligand binding, ErbB proteins homo- or heterodimerize with other members of the ErbB family to activate downstream signaling pathways that regulate proliferation, growth, and differentiation (Riese & Stern 1998). Aberrant expression of the EGFR and/or hyperactivation of this receptor have been associated with the pathogenesis and progression of different types of cancers (Ito *et al.* 2001, Normanno *et al.* 2006). Growth hormone was shown to modulate the expression of EGFR in the liver (Jansson *et al.* 1988, Gonzalez *et al.* 2010). EGFR mRNA levels and epidermal growth factor (EGF) binding to liver were reported to be more efficiently induced by a pulsatile pattern of plasma GH concentration (Jansson *et al.* 1988, Ekberg *et al.* 1989, Kashimata *et al.* 1989). However, results of these studies are controversial since other authors found no differences on the induction of EGFR mRNA or EGF binding when the hormone was administered by intermittent injections or continuous infusion to hypophysectomized rats (Johansson *et al.* 1989). Moreover, we have recently demonstrated that transgenic mice overexpressing GH, which exhibit a continuous plasma GH pattern (Norstedt & Palmiter 1984), show increased EGFR expression (Miquet *et al.* 2008, González *et al.* 2010).

While studies regarding GH concentration patterns and EGFR expression in the liver have been performed, the consequent effects on EGFR signaling have not been analyzed. Increased EGFR liver content not necessarily implies up-regulation of EGF signaling. Actually, transgenic mice overexpressing GH showed elevated EGFR protein levels in the liver but activation upon ligand stimulation was diminished for some of its signaling cascades (González *et al.* 2010, Díaz *et al.* 2012).

Therefore, considering the growing interest in developing sustained delivery systems for GH administration, the potential oncogenic properties of this hormone and its likely dimorphic

regulation of a receptor widely involved in cancer, the EGFR, the aim of this study was to analyze the effects of different GH administration protocols on EGFR expression, signaling and induction of mitogenic mediators in the liver of normal mice.

Materials & Methods

Reagents

Highly purified porcine growth hormone was obtained from Zamira Life Sciences Pty Ltd. (Knoxfield, Australia). Recombinant human EGF was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA.). PVDF membranes, high performance chemiluminescence film and enhanced chemiluminescence (ECL)-Plus were from Amersham Biosciences (Piscataway, NY, USA). Acrylamide, bis-acrylamide and TEMED were obtained from Bio-Rad Laboratories (Hercules, California, USA). Secondary antibodies conjugated with HRP, antibodies anti-MUPs, anti-EGFR, anti-CIS and anti-STAT5 were purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA). Antibody anti-phospho-STAT5a/b Tyr694/699 was from Millipore (Billerica, MA, USA). Antibodies anti-phospho-AKT Ser473, anti-AKT, anti-p44/42 MAP kinase (anti-ERK1/2), anti-phospho-p44/42 MAP kinase Thr202/Tyr204 (anti-phospho-ERK1/2), anti-phospho-STAT3 Tyr705, anti-phospho-EGFR Tyr845, anti-c-Myc and anti-c-Fos were from Cell Signaling Technology Inc. (Beverly, MA, USA). Bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific, Pierce Protein Research Products (Rockford, IL, USA). All other chemicals were of reagent grade.

Animals

Swiss-Webster female and male adult animals (3–4 months old, body weight: 26-30g) were used. The mice were housed 3–5 per cage in a room with controlled light (12 h light/day) and temperature (22±2 ºC). Sentinel animals were tested for all major murine pathogens and the results

of the tests were uniformly negative. The animals had free access to nutritionally balanced diet 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.

Animal treatments

GH-treatment: the hormone was administered to Swiss-Webster mice during five days under two different protocols: i) subcutaneous (s.c.) injections of porcine GH (pGH) (0.5 μg/g of body weight) twice a day and ii) s.c. continuous infusion with an osmotic minipump (model 1007D, Alzet) delivering the hormone in a dose 1 μg/g of body weight per day (Jansson *et al.* 1988, Karlsson *et al.* 1999). Protocols for GH-treatment were adapted from previous research works (Jansson *et al.* 1988, Ekberg *et al.* 1989). Control animals for each group were treated in a similar fashion but saline was administered instead.

Acute EGF stimulation: Mice were fasted for 6 h prior to intraperitoneal injection with recombinant human EGF at 2 mg/kg BW in 0.9% w/v NaCl. Animals were euthanized 10 minutes after EGF administration (González *et al.* 2010) to study phosphorylation of EGF signaling mediators, or after 30 min or 1 h to study the expression of early genes involved in the induction of cell cycle progression. Control mice were injected with saline to evaluate basal conditions. Urine was collected at the time of sacrifice and the livers were removed and stored frozen at -70ºC until homogenization.

Processing of urine samples

Urine samples were processed immediately after being obtained. They were centrifuged at 8,800 g for 3 minutes at 4°C. The supernatant was recovered and diluted in a ratio 1/3 in 0.06 mol/l Tris, 0.001 mol/l EDTA (pH 7.4) buffer. An aliquot of each sample was diluted in Laemmli buffer, boiled for 5 min and stored at –20ºC until electrophoresis. Samples were subjected to SDS-PAGE

using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Gels were stained with Coomasie Blue.

Preparation of liver extracts

Liver samples were homogenized at the ratio 0.1g/ml as previously described (González *et al.* 2010, Díaz *et al.* 2012). Protein concentration of supernatants was determined using a BCA protein assay kit and samples were prepared for immunoblotting (González *et al.* 2010, Díaz *et al.* 2012). For immunoprecipitation, aliquots of solubilized liver containing 4 mg of protein were incubated overnight at 4ºC with anti-CIS antibody and subsequently processed as previously described (González *et al.* 2010).

Immunoblotting

Samples were subjected to SDS-PAGE. Electrotransference of proteins from gel to PVDF membranes, incubation with antibodies, reprobing, detection and quantification of band intensity were performed as already described (González *et al.* 2010, Díaz *et al.* 2012).

RNA Isolation and quantitative RT-PCR

Total RNA from 50 mg liver tissue was extracted with TRIzol (Reagent, Life Technologies, Grand Island, New York, USA) according to the manufacturer's instructions. The purity of RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the integrity was controlled by electrophoresis on 1% agarose gel. 2 μg of total RNA were used for reverse transcription reaction in a 20 μl reaction using M-MLV reverse transcriptase (Promega, 200 U/μl) and random hexamer primers (Biodynamics) (Frungieri *et al.* 2002).

For quantitative real-time PCR, cDNA was amplified in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using SYBR Green Master Mix Reagent (Applied Biosystems). The forward (F) and reverse (R) primers used were: EGFR: F:

ATAGTGGTGGTGGCCCTTGG; R: GAGGTTCCACGAGCTCTCTCT; c-myc: F: TCACCAGCACAACTACGCCG; R: TGCTTCAGGACCCTGCCACT; c-fos: F: CCGACTACGAGGCGTCATCC, R: CTGCGCAAAAGTCCTGTGTGT; cyclophilin A: F: GCGTCTCCTTCGAGCTGTT; R: AAGTCACCACCCTGGCAC. Cyclophilin A was chosen as the housekeeping gene. Reaction data were collected and analyzed by the complementary computer software (Sequence Detection Software, Applied Biosystems, Version 1.3). For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (Pfaffl 2001) was applied. An expression ratio was determined for each sample by calculating $(\text{Etarget})^{\Delta Ct(\text{target})}/(\text{Ecyclophilin})^{\Delta Ct(\text{cyclophilin})}$, where E is the efficiency of the primer set and $\Delta Ct = Ct_{(\text{normalization cDNA})} - Ct_{(\text{experimental cDNA})}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log microliters of cDNA per reaction vs. Ct value ($E = 10^{-(1/\text{slope})}$). Efficiencies of 2 ± 0.1 were considered optimal.

Statistical analysis

Experiments were performed analyzing all groups of animals in parallel, *n* representing the number of different individuals used in each group. Results are presented as mean ± SEM 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 only two groups were analyzed. Data were considered significantly different if $p < 0.05$.

Results

Effects of different GH administration protocols on the expression of proteins regulated by plasma GH concentration patterns

Previous studies have demonstrated that intermittent injections and continuous administration of GH produce differential plasma concentrations patterns of the hormone and effects; however, those studies were performed in hypophysectomized animals (Jansson *et al.* 1988, Ekberg *et al.* 1989, Waxman *et al.* 1991). In the present study, non-hypophysectomized mice were studied. In normal rodents, as it occurs in several species, GH secretion is sexually dimorphic (Jansson *et al.* 1985; MacLeod *et al.* 1991, Veldhuis & Bowers 2003). A key difference between male and female GH profiles is the sustained interpulse interval of little or no detectable circulating GH characteristic of adult males (Jansson *et al.* 1985, MacLeod *et al.* 1991, Jaffe *et al.* 1998). Normal female and male mice also have a different endocrine and metabolic background. To study the effects of different GH concentration patterns in normal mice, supraphysiological GH doses were exogenously administered in an intermittent or continuous fashion. Then, the expression of proteins regulated by the GH plasma concentration patterns were assessed in order to establish a relationship between the GH administration protocols and the GH plasma concentration patterns produced by the treatments. For this purpose, MUPs (mayor urinary proteins) and CIS (cytokine-inducible SH2-domain containing protein) liver content and MUPS urine concentration were determined in GH-treated and control mice.

The MUPs are acidic protein isoforms with a molecular mass between 19- 21 kDa (Cavaggioni & Mucignat-Caretta 2000). These pheromone-binding proteins are synthesized in the liver and released through the kidney into the urine. The secretory pattern of GH regulates MUPs gene expression; induction of MUPs mRNA requires pulsatile occupancy of GH receptors, while continuous GH concentration pattern represses MUPs gene expression (Norstedt & Palmiter 1984, Johnson *et al.* 1995). To show the difference in MUPs hepatic content between male and female mice, samples from both sexes were analyzed in parallel (Supplementary Figure 1 A). Intermittent administration of GH to female mice produced an increase in MUPs liver content (Fig. 1 A); however, this GH administration protocol caused no effects on MUPs expression in male mice (Fig. 1 B). On the contrary, continuous administration of the hormone had no effects on MUPs liver

expression in female mice (Fig. 1 C) but caused a decrease in MUPs liver content in male mice (Fig. 1 D). To confirm the effects of exogenously administered GH on MUPs production, concentration of these proteins was determined in urine. According to results obtained for MUPs liver content, MUPs urine concentration increased in female mice treated with intermittent injections of GH (Fig. 1 E) while diminished in male mice that received continuous administration of the hormone (Fig. 1 F). The absence of effects on MUPs expression observed in male mice injected with GH could be explained by the already high MUPs levels induced by the endogenous secretion pattern of the hormone. In the case of female mice, no effects on MUPs expression were found in animals treated with GH delivered by the osmotic pumps because MUPs expression is already low.

The liver content of the suppressor of cytokine signaling CIS also varies depending on GH plasma concentration patterns (Karlsson *et al.* 1999). CIS is induced by a continuous pattern of GH concentration (Ram & Waxman, 2000). Consequently, its expression decreased in female mice that received intermittent injections of GH respect to controls (Fig. 1 G), but increased in male mice administered with GH in a continuous fashion compared to non-treated male mice (Fig. 1 H). A tendency to decreased CIS levels was also evidenced when GH was administered by intermittent injections to male mice; however, the difference was not significant (Supplementary Fig. 1B). Continuous administration of GH to female mice induced a slight and not significant increase in CIS hepatic levels (Supplementary Fig. 1C).

Therefore, a pulsatile pattern of GH concentration was evidenced in female mice exogenously treated with intermittent injections of GH while a continuous pattern of GH circulating levels was demonstrated to be caused by sustained delivery of GH in male mice. For this reason, the effects of pulsatile GH plasma concentration patterns on EGFR expression and signaling were studied in female mice that received intermittent injections of GH or saline. On the other hand, the effects of continuous concentration patterns of the hormone were analyzed in male mice by implantation of sustained delivery systems for continuous release of the hormone or saline.

Additionally, control assays implying intermittent administration of GH to male mice and continuous delivery of the hormone in female mice were performed.

EGFR expression, activation and signaling vary with the pattern of plasma GH concentrations

The effects of plasma GH patterns on hepatic EGFR mRNA levels and EGFR protein content were determined in the liver of female mice that received intermittent treatment with GH and in the liver of male mice that received continuous administration of the hormone (Fig. 2 A- D). EGFR mRNA levels increased in the liver of mice that received intermittent GH injections, but results were not statistically different from those of non-treated mice (Fig. 2 A). On the contrary, GH administration by osmotic pumps provoked a decline of EGFR mRNA levels (Fig. 2 B). EGFR protein content was analyzed in solubilized liver from GH-treated and control mice that subsequently received acute stimulation with EGF. Immunoblotting analysis showed that the protein content of EGFR was significantly increased by a pulsatile plasma GH pattern (Fig. 2 C), while continuous circulating levels of GH decreased EGFR protein abundance (Fig. 2 D) in accordance with results for EGFR mRNA determination.

Ligand-induced EGFR dimerization leads to receptor autophosphorylation at tyrosine residues. Phosphotyrosine residues allow the recruitment of specific partners to activate different downstream pathways, including Ras/MAPK, PI3K/Akt and signal transducer and activator of transcription (STATs) pathways (Wells 1999, Henson & Gibson 2006, Normanno *et al.* 2006). EGFR is augmented in transgenic mice overexpressing growth hormone, however, this does not result in an increased response to EGF (González *et al.* 2010). To ascertain the effects of the different GH plasma patterns on EGF-induced EGFR activation, EGFR phosphorylation on tyrosine 845 was assessed in the liver of GH-treated and non-treated mice after acute stimulation with EGF (Fig. 2 E and F). In accordance with the changes described for EGFR hepatic content, EGF-induced receptor phosphorylation levels increased in mice that received subcutaneous injections of GH

compared with untreated mice (Fig. 2 E), but decreased in mice that received continuous administration of GH respect to their controls (Fig. 2 F). The analysis of the ratio between EGFR phosphorylation levels and EGFR liver content showed that increased or decreased EGFR phosphorylation levels could be attributed to the augmented or reduced hepatic protein amount, respectively (Fig. 2 G and H).

The MAP kinases Erk1/2 have been extensively associated with the promotion of cell proliferation. To ascertain the effects of different plasma GH concentration patterns on the regulation of EGF-induced Erk1/2 activation, Erk1/2 phosphorylation was determined in liver of mice that received intermittent or sustained administration of GH followed by the acute stimulation with EGF or saline. In accordance with results described for EGFR, phosphorylation levels of Erk1/2 were increased in female mice treated with GH injections compared with untreated mice (Fig. 3 A), but decreased in liver of male mice that received continuous administration of GH (Fig. 3 B). No differences in the protein levels of Erk1/2 were observed for any of the treatments (Fig. 3 A and B).

The activation of EGFR also results in Akt phosphorylation of its two main and activating phosphorylation sites, Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain (Fresno Vara *et al.* 2004, Osaki *et al.* 2004). Once activated, Akt promotes cell survival and proliferation. Akt protein content and phosphorylation of its activating residues were determined in liver of mice that received intermittent or sustained administration of GH (Fig. 3 C- F). In accordance with results found for Erk1/2, Akt activation was augmented in the liver of female mice that received intermittent administration of GH (Fig. 3 C and E) while diminished in the liver of male mice that received the hormone in a continuous fashion (Fig. 3 D and F). The protein abundance of the kinase did not significantly vary after the different GH treatments (Fig. 3 C- F).

EGFR activation also results in STATs activation (Guren *et al.* 2003, Quesnelle *et al.* 2007), which have been associated with development and progression of many malignancies (Buettner *et al.* 2002, Quesnelle *et al.* 2007). EGF-induced STAT3 phosphorylation was compared in mice that

received the different GH administration protocols and in their respective untreated control mice (Fig. 4). In accordance with findings described for Erk1/2 and Akt activation, EGF-induced STAT3 phosphorylation increased upon a pulsatile GH concentration pattern (Fig. 4 A), while diminished when the GH plasma concentration pattern was continuous (Fig. 4 B). However, protein content was not modified upon the different GH administration protocols (Fig. 4 A and B). Similar results were found for STAT5 activation when EGF-induced phosphorylation and protein levels were studied in mice that received GH treatments and in their respective controls (Fig. 4 C and D).

EGFR protein levels and EGF-induced activation of Erk1/2 and Akt were also determined, as a control, in female mice that received continuous administration of GH and in male mice injected with intermittent doses of the hormone. Continuous administration of GH to female mice had no effects on EGFR liver content, while GH injections induced a slight but non-significant increase in hepatic EGFR content (Fig 5 A and B). In accordance with the absence of effects on EGFR levels, neither continuous administration of GH to female mice nor intermittent injection of GH to normal male mice induced changes in Erk1/2 or Akt phosphorylation levels in response to EGF (Fig. 5 C-F). The absence of effects of GH administered by the osmotic pumps to female mice on EGFR expression and EGF signaling correlates with the lack of effects of the treatment observed for MUPS expression (Fig 1 C). Similarly, intermittent GH injections to male mice had no effects on EGFR hepatic levels and EGF signaling as wells as on MUPs expression (Fig 1B). In both cases, exogenous administration of GH does not seem to induce a net change respect to endogenous GH concentration patterns.

The pattern of plasma GH concentrations affects EGF-induction of proteins involved in cell cycle promotion

As a consequence of cellular stimulation with several mitogenic signals including the EGF, c-myc expression and activation is induced, mainly via the MAPK/ERK pathway (Curran *et al.* 1985). Considering the differential effects on EGFR expression and signaling observed in mice

treated with intermittent injections of GH or with a sustained delivery system for GH administration, c-myc induction upon EGF stimulation was assessed to elucidate the possible differential effects of plasma GH concentration patterns on EGF-induced proliferation in liver. For this purpose, the kinetic of c-myc mRNA and protein expression upon exogenous administration of EGF for different times was first evaluated in the liver of female and male mice with no GH treatment. No sex differences were observed, both mRNA and protein expression showed maximal induction 1 h after EGF injection (Fig. 6 A and B); therefore, this time period was selected to evaluate the effects of different GH concentration patterns. In accordance with variations described for EGFR expression and signaling, EGF-induced c-myc mRNA and protein content were increased by the pulsatile GH concentration pattern induced in female mice by GH injections (Fig 6 C and E) while decreased by the continuous GH concentration pattern provoked in male mice by sustained delivery of the hormone (Fig. 6 D and F).

EGF-induced expression of the oncogene c-fos was also analyzed in the liver of mice that received GH by different modes of administration. Kinetic of c-fos transcription and transduction upon EGF stimulation was first assessed in the liver of non-GH treated female and male mice. c-Fos mRNA levels reached the maximum 30 minutes after EGF injection, while protein content attained higher levels 1 h after EGF administration (Fig. 7 A and B) in both sexes. Considering the data from kinetic studies, c-fos mRNA and protein content were measured in the liver of non-treated and GH-treated mice, in basal conditions and after acute stimulation with EGF. In accordance with previous results, both hepatic c-fos mRNA and protein levels were increased by the pulsatile GH concentration pattern (Fig. 7 C and E) while decreased by the continuous plasma GH pattern (Fig. 7 D and F).

360 **Discussion**

361 Currently, there is increasing interest in developing delivery systems that allow sustained
362 release of GH to prolong administration intervals of the hormone during a chronic treatment.
363 However, plasma GH concentration patterns induce differential physiological actions in several
364 species. For this reason, continuous administration of the hormone must be validated regarding not
365 only its efficacy but also its safety.

366 In humans, only a few studies have compared the effects of continuous subcutaneous infusion
367 versus daily subcutaneous injections of GH. The studies were mainly focused on the actions over
368 longitudinal growth, body weight, body composition and metabolism and revealed some differences
369 regarding IGF-I and IGFBP-3 plasma levels, glucose tolerance (Johansson *et al.* 1996) and lipid
370 metabolism, but the effects on longitudinal growth and body composition were not of critical
371 significance (Laursen *et al.* 2001). In animals, the physiological importance of GH concentration
372 patterns has been extensively described. Actually, GH concentration patterns have differential
373 effects on the hepatic expression of numerous genes (Norstedt & Palmiter 1984; Waxman *et al.*
374 1991, Waxman & O'Connor 2006) and GH administered in a pulsatile manner induces growth and
375 insulin-like growth factor I (IGF-I) generation more effectively than continuous administration
376 (Clark & Robinson 1988, Udy *et al.* 1997).

377 While several studies have been carried out to establish the efficacy of continuous
378 administration of GH, there are not enough studies to determine the security of this treatment
379 compared to GH intermittent administration. Among the main problems associated with long term
380 GH-treatment is the increased tendency to develop tumors. The expression of the epidermal growth
381 factor receptor, broadly involved in the pathogenesis of different types of tumors, varies depending
382 on GH plasma levels. EGFR plays an essential role in the development of epithelial cells but also in
383 tumors of epithelial cell origin (Singh & Harris 2005). Therefore, the objective of this study was to
384 investigate how different GH plasma patterns, induced by different administration protocols, affect
385 EGFR expression, signaling and induction of mitogenic mediators in the liver of normal mice.

The effects of GH plasma levels on EGFR expression have been extensively studied. GH administration was described to induce the expression of EGFR in the liver while hypophysectomy reduced the hepatic EGF receptor mRNA concentration in rats (Ekberg *et al.* 1989). In accordance, EGFR liver expression is increased in transgenic mice over-expressing growth hormone but decreased in GH-receptor knock-out mice (Miquet *et al.* 2008, González *et al.* 2010). However, studies concerning the effects of plasma growth hormone pattern on EGFR expression are still controversial. In rodents, EGFR expression is higher in males than in females as evidenced by specific binding of labeled EGF to purified liver membranes, EGFR mRNA determination and immunoblotting from solubilized liver (Ekberg *et al.* 1989, Johansson *et al.* 1989, Miquet *et al.* 2013). Furthermore, in hypophysectomized rats of both sexes, intermittent GH treatment enhanced hepatic EGF receptor mRNA concentrations to normal male levels, while continuous GH administration was less effective or did not have any effects at all (Ekberg *et al.* 1989, Kashimata *et al.* 1989). On the contrary, other authors found no differences between intermittent or continuous administration of hGH on the induction of EGFR mRNA or EGF binding when administered to hypophysectomized male and female rats (Johansson *et al.* 1989). Moreover, increased GH levels with a continuous pattern in transgenic mice overexpressing GH also derives in the up-regulation of liver EGFR expression (Miquet *et al.* 2008, González *et al.* 2010). These genetically modified mice present high GH levels during the entire lifespan of the animals and many physiological and endocrine alterations may be associated to GH overexpression; therefore, it is difficult to dissect the mechanisms involved in EGFR expression and signaling changes. For this reason, the influence of GH plasma patterns was studied in normal mice supplemented with the hormone for a short period. Subcutaneous injections of GH increased hepatic EGFR content in female mice liver, while continuous administration of the hormone to male mice reduced hepatic receptor mRNA levels and protein content. Therefore, results support the relevance of the GH concentration pattern as an important determinant of EGFR expression. In accordance, EGFR levels did not change in female mice liver after continuous administration of GH or in male mice that received intermittent

injections of the hormone. As previously discussed for MUPs expression, EGFR was not significantly induced in male mice injected with GH because EGFR liver content is already high due to the endogenous secretion pattern of the hormone. On the contrary, reduction of EGFR levels were not evidenced in female mice liver after continuous GH treatment probably because content of the receptor is already low compared to male mice (Miquet *et al*. 2013).

Continuous GH plasma levels reduced EGFR protein content and mRNA levels in liver. In contrast, EGFR protein levels were found to be increased in the liver of female mice that received the intermittent treatment with GH but quantitative RT-PCR of the EGFR showed a tendency to higher levels which was non-significantly different from control mice. The discrepancy between immunoblotting and quantitative PCR determinations of EGFR might depend on possible effects of GH on EGFR turnover. GH and prolactin (PRL), a family related protein, were shown to control EGFR turnover by phosphorylation on serine/threonine residues (Huang *et al.* 2003, 2004, 2006) which delay EGFR down-regulation and therefore potentiate acute EGF-induced signaling (Huang *et al.* 2003, 2004, 2006, Li *et al.* 2008). Besides inducing EGFR expression, intermittent administered GH could provoke retardation of EGFR down-regulation, thus increasing EGFR protein content to a greater extent than EGFR mRNA levels.

Results from the present study demonstrated that the effects of GH administration on liver EGFR content directly correlated with effects on EGF-induced signalling. Such effects were not necessarily the expected results as we had previously observed that transgenic mice over-expressing GH showed an increase in hepatic EGFR content but not in EGF signaling (González *et al.* 2010). Transgenic mice overexpressing GH present high GH levels during their lifespan which triggers several mechanisms to down-regulate EGF signal through the STATs and the PI3K/Akt pathways (González *et al.* 2010, Díaz *et al.* 2012). Short-term treatment with GH would not induce such compensatory mechanisms, consequently, changes in EGF signaling directly correlated with modifications in EGFR liver content.

Considering the role of EGFR in cell proliferation and tumor development, EGF-induced c-myc and c-fos protein and mRNA content were determined. c-Myc is a pleiotropic transcription factor that promotes growth and expansion of somatic cells, its activation results in up-regulation of cyclins with concomitant down-regulation of cell cycle inhibitors like p21. c-Myc is an important proto-oncogene, found to be up-regulated in many types of cancers (Soucek *et al.* 2008, Lin *et al.* 2010). c-Fos is a transcription factor that belongs to the family of immediate early genes which is up-regulated in response to several mitogenic signals, including EGF (Curran *et al.* 1985). Increased expression of c-fos has been associated with cancer development, including hepatocarcinoma (Yuen *et al.* 2001, Liu *et al.* 2012). In accordance with effects of plasma GH pattern on EGF signal, induction of c-myc was higher in female mice that received intermittent injections of GH compared to non-treated animals; and c-myc induction was lowered by the continuous GH pattern in male mice. Similar results were described when c-fos expression and protein content were determined.

In conclusion, a pulsatile GH concentration pattern, produced by intermittent injections of the hormone, induced the up-regulation of liver EGFR content, which resulted in an increased response to EGF in female mice, but non-significant effects on EGFR expression and signaling in male mice. On the contrary, continuous GH concentration patterns, induced by sustained administration of the hormone, had no effects on EGFR expression or EGF-induced response in female mice liver, but decreased EGFR expression and activation of EGF-induced signaling mediators in male mice tissue, resulting in diminished pro-mitogenic effects. Results would suggest the beneficial use of sustained delivery systems that allow continuous GH plasma patterns in terms of treatment security relating to EGFR-mediated protumorogenic adverse effects. Future studies should be performed in GH-deficient (GHD) animal models to ascertain the beneficial use of those systems for the treatment of GHD associated states. To extend this conclusion to humans, species differences in GH secretion patterns should be considered and studies on the effects of different plasma GH patterns should be carried out in humans. Moreover, assessment of other GH-regulated proteins like serum IGF-I and IGFBP-3 levels, which have been described as predictors of hepatocarcinoma

development, should also be considered in order to determine the potential adverse effects of different administration protocols on tumor promotion.

Declaration of interest

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

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626 **Figure Legends:**

627 **Figure 1: MUPs and CIS liver content and MUPs urine concentration in GH-treated**
628 **and control mice.** Solubilized liver samples from female and male mice treated with intermittent
629 injections (intGH) or continuous delivery of GH (contGH) and their respective controls were
630 subjected to immunoblotting analysis to determine MUPs (21 kDa) hepatic content (A, B, C, D).
631 Reprobing with anti-actin antibody demonstrated similar protein loading in all lanes. Urine samples
632 from female mice treated with intermittent injections of GH, male mice receiving continuous
633 infusion of GH and their respective controls were analyzed by SDS-PAGE to assess urine MUPs
634 concentration (E and F). CIS (32 kDa) was immunoprecipitated from solubilized liver from female
635 and male mice that received intermittent or continuous GH treatment, respectively, and assessed by
636 immunoblotting with anti-CIS (G and H). Quantification was performed by scanning densitometry
637 and expressed as fold change vs. values measured for GH-untreated normal mice. Data are the mean
638 ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote
639 significant difference at $p < 0.05$. Representative western blots are shown.
640
641 **Figure 2: Hepatic EGFR mRNA expression, protein content and phosphorylation.** Liver
642 samples from female mice treated with intermittent injections of GH (intGH), male mice receiving
643 continuous delivery of GH (contGH) and their respective controls were analyzed by qRT-PCR to
644 determine EGFR mRNA levels (A and B). Solubilized liver from GH-treated and control mice that
645 subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to
646 immunoblot analysis to determine EGFR (170 kDa) liver content (C and D) and EGFR Tyr845
647 phosphorylation levels (E and F). Membranes were reprobed to asses actin content and demonstrate
648 uniform protein loading in all lanes. Immunoblots were scanned and quantification was performed
649 by densitometry. Data resulting form quantification analysis were used to calculate the pY845
650 EGFR/EGFR ratio (G and H). Results were expressed as fold change vs. values measured for non-

651 treated normal mice (A and B) or EGF-stimulated GH-untreated mice (C, D, E, F, G and H). Data
652 are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters
653 denote significant difference at $p< 0.05$. Representative western blots are shown.

654

655 **Figure 3: Hepatic Erk1/2 and Akt phosphorylation and protein content.** Solubilized liver
656 from female mice treated with intermittent injections of GH (intGH), male mice receiving
657 continuous infusion of GH (contGH) and their respective controls that subsequently received acute
658 stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to immunoblot analysis to
659 determine Erk1/2 (44/42 kDa) phosphorylation levels and its protein content (A and B); Akt (62
660 kDa) phosphorylation levels at Ser473 (C and D), at Thr308 (E and F) and its protein content (C, D,
661 E and F). Reprobing with anti-actin antibody demonstrated equal protein loading in all
662 lanes.Quantification was performed by scanning densitometry and expressed as fold change vs.
663 EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets
664 (n) of individual animals. Different letters denote significant difference at $p< 0.05$. Representative
665 western blots are shown.

666

667 **Figure 4: STAT3 and STAT5 phosphorylation and protein content in the liver.**
668 Solubilized liver from female mice treated with intermittent injections of GH (intGH), male mice
669 that received continuous delivery of GH (contGH) and their respective controls that subsequently
670 received acute stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to immunoblot
671 analysis to determine phosphorylation levels of STAT3 (86-91 kDa) (A and B) and STAT5 (92
672 kDa) (C and D) as well as their protein content (A, B, C, and D). Reprobing with anti-actin antibody
673 demonstrated uniform protein loading in all lanes.Quantification was performed by scanning
674 densitometry and expressed as fold change vs. EGF-stimulated GH-untreated mice. Data are the

675 mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote
676 significant difference at $p< 0.05$. Representative western blots are shown.
677
678 **Figure 5: Hepatic EGFR content, Erk1/2 and Akt phosphorylation and protein**
679 **detection.** Solubilized liver from female mice receiving continuous delivery of GH (contGH), male
680 mice treated with intermittent injections of GH (intGH), and their respective controls which
681 subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were analyzed by
682 immunoblotting to determine EGFR protein levels (A and B), Erk1/2 and Akt phosphorylation and
683 protein content (C, D, E and F). Membranes were reprobed to asses actin content and demonstrate
684 equal protein loading in all lanes.Quantification was performed by scanning densitometry and
685 expressed as fold change vs. EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the
686 indicated number of subsets (n) of individual animals. Different letters denote significant difference
687 at $p< 0.05$. Representative western blots are shown.
688
689 **Figure 6: Hepatic c-Myc expression and protein content.** Liver samples from mice non-
690 stimulated and stimulated with EGF during different time periods were analyzed by qRT-PCR and
691 immunoblotting to determine c-myc expression and its protein content induction, respectively (A,
692 B). c–Myc (67 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent
693 injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their
694 respective controls that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (C
695 and D). Protein content of liver samples was analyzed by immunoblotting in GH-treated and control
696 mice that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (E and F).
697 Reprobing with anti-actin antibody demonstrated equal protein loading in all lanes. Immunoblots
698 were scanned and quantification was performed by densitometry. Results were expressed as fold
699 change vs. values measured for EGF-stimulated GH-untreated mice (A, C, D, E, F). Data are the

mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at $p< 0.05$. Representative western blots are shown.

Figure 7: Hepatic c-fos expression and protein content. Liver samples from mice stimulated with EGF during different time periods and their non-stimulated controls were analyzed by qRT-PCR and immunoblotting to determine c-fos expression and its protein content induction, respectively (A, B). c–Fos (62 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their respective controls that received acute stimulation with EGF during 30 min (EGF 30 min) or not (-) (C and D). Protein content of liver samples was analyzed by immunoblotting in GH-treated and control mice that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (E and F). Reprobing with anti-actin antibody demonstrated uniform protein loading in all lanes. Immunoblots were scanned and quantification was performed by densitometry. Results were expressed as fold change vs. values measured for EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at $p< 0.05$. Representative western blots are shown.

Supplementary figure 1: MUPs liver content in control animals and CIS amount in GH-treated and control mice. Solubilized liver samples from female and male mice were subjected to immunoblotting analysis to determine MUPs hepatic content (A). Reprobing with anti-actin antibody demonstrated uniform protein loading in all lanes. CIS was immunoprecipitated from solubilized liver from female and male mice that received continuous or intermittent GH treatment, respectively, and assessed by immunoblotting with anti-CIS (B and C). Quantification was performed by scanning densitometry and expressed as fold change vs. female mice (A) or non-

725 treated normal mice (B and C). Data are the mean ± SEM of the indicated number of subsets (n) of
726 individual animals. Different letters denote significant difference at $p < 0.05$. Representative western
727 blots are shown.

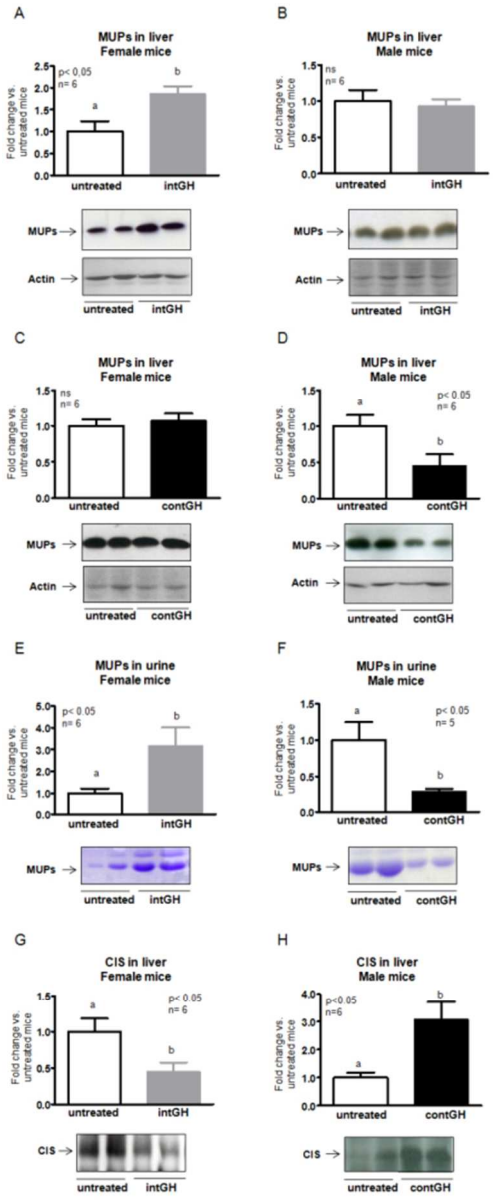

Figure 1: MUPs and CIS liver content and MUPs urine concentration in GH-treated and control mice. Solubilized liver samples from female and male mice treated with intermittent injections (intGH) or continuous delivery of GH (contGH) and their respective controls were subjected to immunoblotting analysis to determine MUPs (21 kDa) hepatic content (A, B, C, D). Reprobing with anti-actin antibody demonstrated similar protein loading in all lanes. Urine samples from female mice treated with intermittent injections of GH, male mice receiving continuous infusion of GH and their respective controls were analyzed by SDS-PAGE to assess urine MUPs concentration (E and F). CIS (32 kDa) was immunoprecipitated from solubilized liver from female and male mice that received intermittent or continuous GH treatment, respectively, and assessed by immunoblotting with anti-CIS (G and H). Quantification was performed by scanning densitometry and expressed as fold change vs. values measured for GH-untreated normal mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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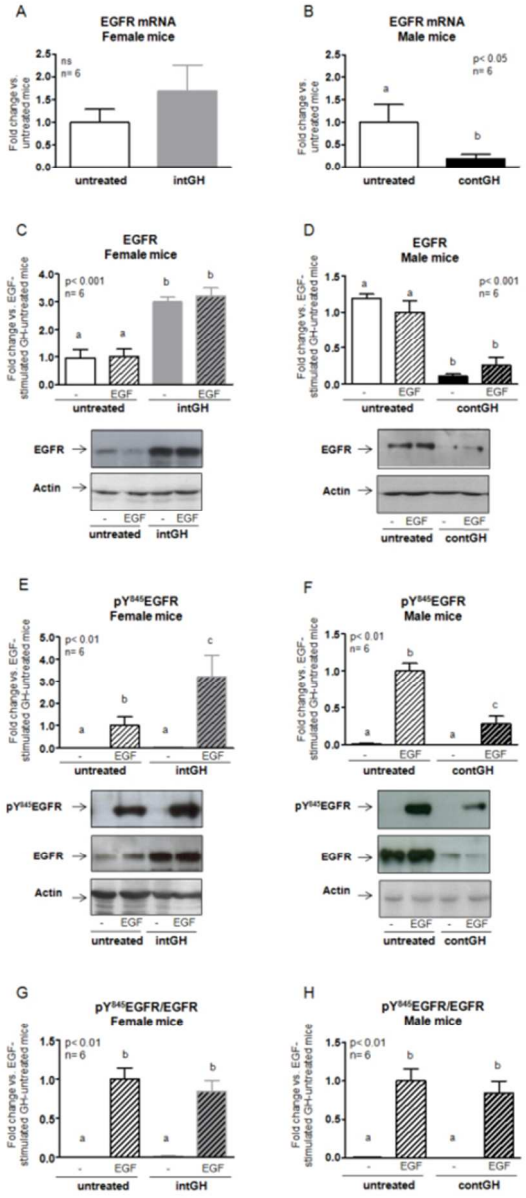

Figure 2: Hepatic EGFR mRNA expression, protein content and phosphorylation. Liver samples from female mice treated with intermittent injections of GH (intGH), male mice receiving continuous delivery of GH (contGH) and their respective controls were analyzed by qRT-PCR to determine EGFR mRNA levels (A and B). Solubilized liver from GH-treated and control mice that subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to immunoblot analysis to determine EGFR (170 kDa) liver content (C and D) and EGFR Tyr845 phosphorylation levels (E and F). Membranes were reprobed to asses actin content and demonstrate uniform protein loading in all lanes. Immunoblots were scanned and quantification was performed by densitometry. Data resulting form quantification analysis were used to calculate the pY845 EGFR/EGFR ratio (G and H). Results were expressed as fold change vs. values measured for non-treated normal mice (A and B) or EGF-stimulated GH-untreated mice (C, D, E, F, G and H). Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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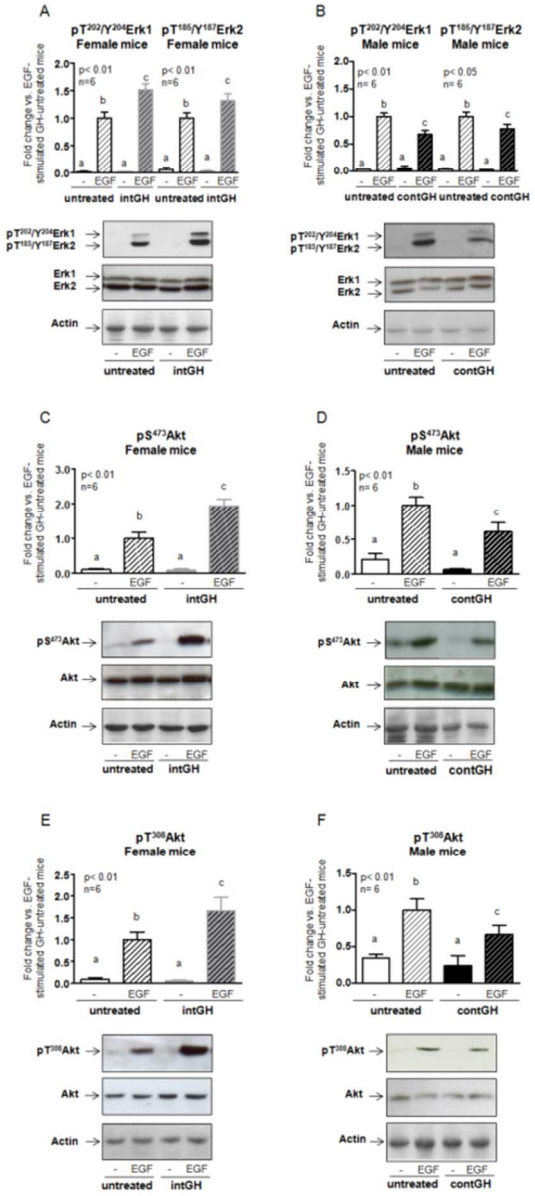

Figure 3: Hepatic Erk1/2 and Akt phosphorylation and protein content. Solubilized liver from female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their respective controls that subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to immunoblot analysis to determine Erk1/2 (44/42 kDa) phosphorylation levels and its protein content (A and B); Akt (62 kDa) phosphorylation levels at Ser473 (C and D), at Thr308 (E and F) and its protein content (C, D, E and F). Reprobing with anti-actin antibody demonstrated equal protein loading in all lanes.Quantification was performed by scanning densitometry and expressed as fold change vs. EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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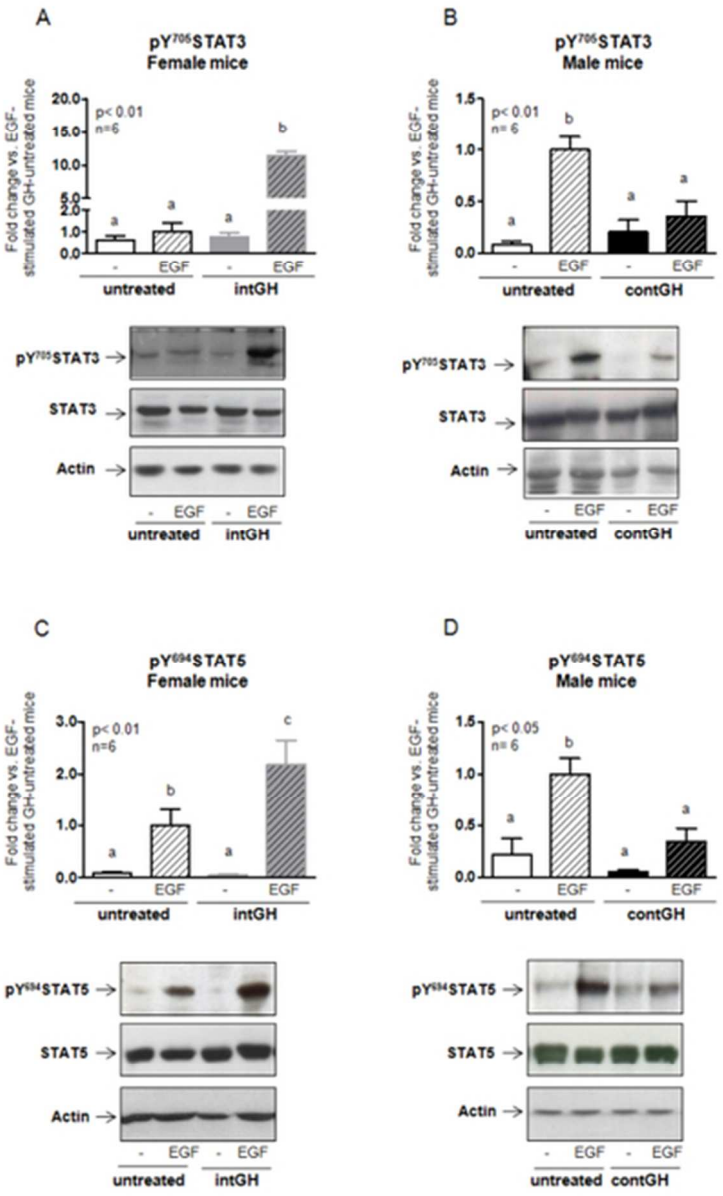

Figure 4: STAT3 and STAT5 phosphorylation and protein content in the liver. Solubilized liver from female mice treated with intermittent injections of GH (intGH), male mice that received continuous delivery of GH (contGH) and their respective controls that subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were subjected to immunoblot analysis to determine phosphorylation levels of STAT3 (86-91 kDa) (A and B) and STAT5 (92 kDa) (C and D) as well as their protein content (A, B, C, and D). Reprobing with anti-actin antibody demonstrated uniform protein loading in all lanes.Quantification was performed by scanning densitometry and expressed as fold change vs. EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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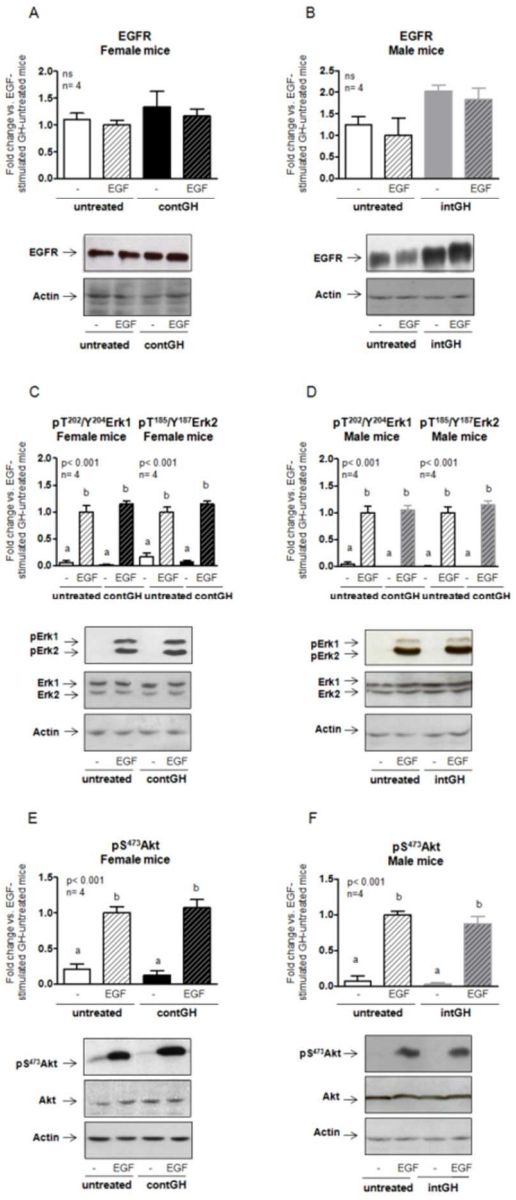

Figure 5: Hepatic EGFR content, Erk1/2 and Akt phosphorylation and protein detection. Solubilized liver from female mice receiving continuous delivery of GH (contGH), male mice treated with intermittent injections of GH (intGH), and their respective controls which subsequently received acute stimulation with EGF for 10 minutes (EGF) or not (-) were analyzed by immunoblotting to determine EGFR protein levels (A and B), Erk1/2 and Akt phosphorylation and protein content (C, D, E and F). Membranes were reprobed to asses actin content and demonstrate equal protein loading in all lanes.Quantification was performed by scanning densitometry and expressed as fold change vs. EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at $p \leq 0.05$. Representative western blots are shown.
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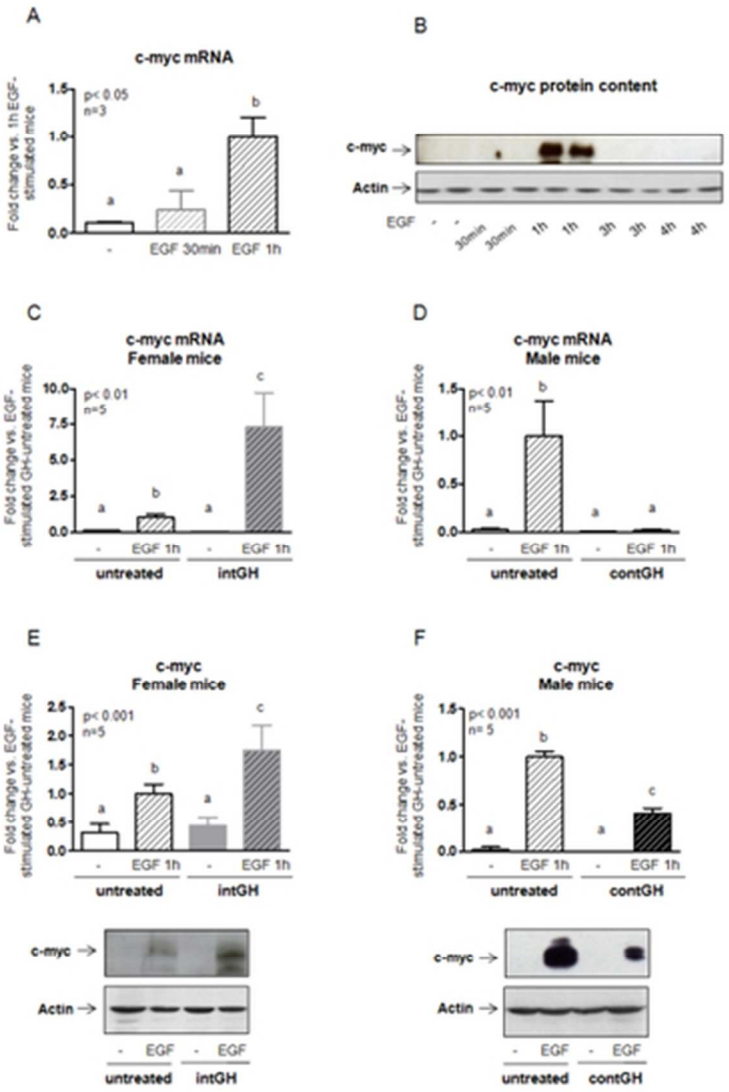

Figure 6: Hepatic c-Myc expression and protein content. Liver samples from mice non-stimulated and stimulated with EGF during different time periods were analyzed by qRT-PCR and immunoblotting to determine c-myc expression and its protein content induction, respectively (A, B). c–Myc (67 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their respective controls that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (C and D). Protein content of liver samples was analyzed by immunoblotting in GH-treated and control mice that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (E and F). Reprobing with anti-actin antibody demonstrated equal protein loading in all lanes. Immunoblots were scanned and quantification was performed by densitometry. Results were expressed as fold change vs. values measured for EGF-stimulated GH-untreated mice (A, C, D, E, F). Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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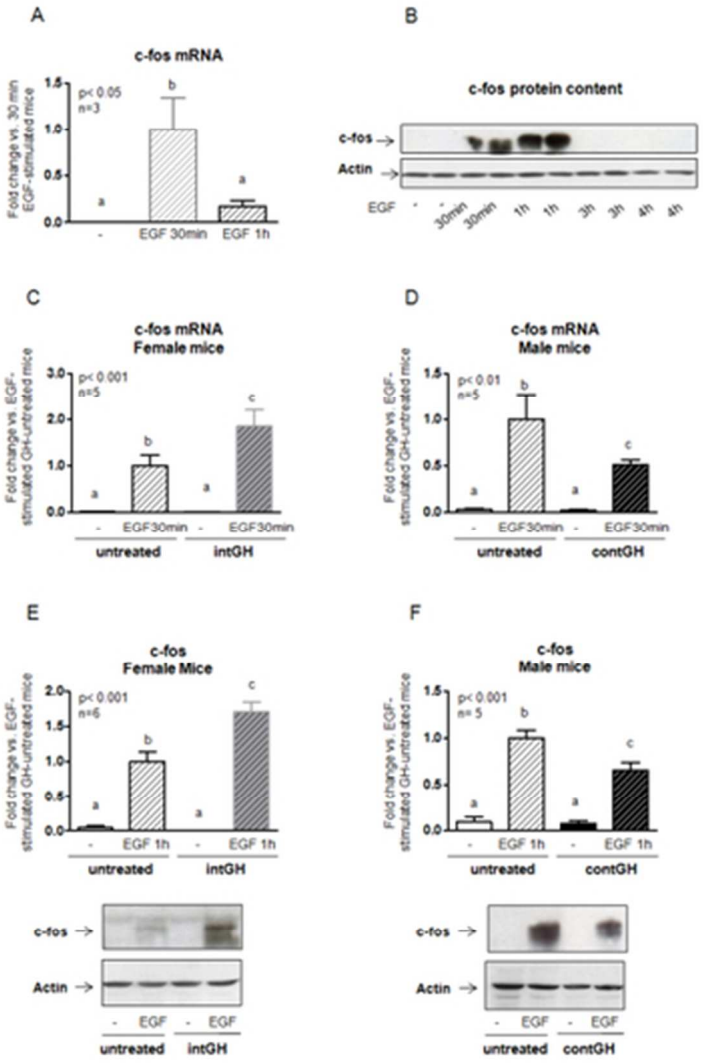

Figure 7: Hepatic c-fos expression and protein content. Liver samples from mice stimulated with EGF during different time periods and their non-stimulated controls were analyzed by qRT-PCR and immunoblotting to determine c-fos expression and its protein content induction, respectively (A, B). c–Fos (62 kDa) expression was analyzed by qRT-PCR in female mice treated with intermittent injections of GH (intGH), male mice receiving continuous infusion of GH (contGH) and their respective controls that received acute stimulation with EGF during 30 min (EGF 30 min) or not (-) (C and D). Protein content of liver samples was analyzed by immunoblotting in GH-treated and control mice that received acute stimulation with EGF during 1 hour (EGF 1h) or not (-) (E and F). Reprobing with anti-actin antibody demonstrated uniform protein loading in all lanes. Immunoblots were scanned and quantification was performed by densitometry. Results were expressed as fold change vs. values measured for EGF-stimulated GH-untreated mice. Data are the mean ± SEM of the indicated number of subsets (n) of individual animals. Different letters denote significant difference at p≤ 0.05. Representative western blots are shown.
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