RESEARCH

Growth hormone upregulates the pro-tumorigenic galectin 1 in mouse liver

María L Bacigalupo[1,*], Verónica G Piazza[1,*], Nadia S Cicconi[1], Pablo Carabias[1], Andrzej Bartke[2], Yimin Fang[2], Ana I Sotelo[1], Gabriel A Rabinovich[3], María F Troncoso[1,†] and **Johanna G Miquet[1,†]**

[1]Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Química y Fisicoquímica Biológicas, Buenos Aires, Argentina
[2]Department of Internal Medicine, Geriatrics Research, Southern Illinois University School of Medicine, Springfield, Illinois, USA
[3]Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas, and Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence should be addressed to M F Troncoso or J G Miquet: fernanda@qb.ffyb.uba.ar or jmiquet@qb.ffyb.uba.ar

*(M L Bacigalupo and V G Piazza contributed equally to this work)

†(M F Troncoso and J G Miquet contributed equally as co-senior authors)

Abstract

Transgenic mice overexpressing growth hormone (GH) spontaneously develop liver tumors, including hepatocellular carcinoma (HCC), within a year. The preneoplastic liver pathology in these mice recapitulates that observed in humans at high risk of developing hepatic cancer. Although increased expression of galectin 1 (GAL1) in liver tissue is associated with HCC aggressiveness, a link between this glycan-binding protein and hormone-related tumor development has not yet been explored. In this study, we investigated GAL1 expression during liver tumor progression in mice continuously exposed to high levels of GH. GAL1 expression was determined by Western blotting, RT-qPCR and immunohistochemistry in the liver of transgenic mice overexpressing GH. Animals of representative ages at different stages of liver pathology were studied. GAL1 expression was upregulated in the liver of GH-transgenic mice. This effect was observed at early ages, when animals displayed no signs of liver disease or minimal histopathological alterations and was also detected in young adults with preneoplastic liver pathology. Remarkably, GAL1 upregulation was sustained during aging and its expression was particularly enhanced in liver tumors. GH also induced hepatic GAL1 expression in mice that were treated with this hormone for a short period. Moreover, GH triggered a rapid increment in GAL1 protein expression in human HCC cells, denoting a direct effect of the hormone on hepatocytes. Therefore, our results indicate that GH upregulates GAL1 expression in mouse liver, which may have critical implications in tumorigenesis. These findings suggest that this lectin could be implicated in hormone-driven liver carcinogenesis.

Key Words

- growth hormone
- galectin 1
- hepatocellular carcinoma
- liver cancer

Endocrine Connections (2019) **8**, 1108–1117

Introduction

Liver cancer is the third most common cause of cancer-related deaths worldwide (1). Hepatocellular carcinoma (HCC) accounts for 90% of liver tumors and it is strongly associated with chronic hepatitis B or C virus infection, alcohol abuse or nonalcoholic steatohepatitis. Regardless of the carcinogenic insult, it usually develops in patients with cirrhosis due to chronic inflammation and advanced fibrosis (2).

Growth hormone (GH) is a major regulator of body growth and metabolism. This hormone is secreted by the pituitary gland and acts directly on target cells by signaling through its membrane-associated receptor.

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

GH also promotes insulin-like growth factor type 1 (IGF1) synthesis, principally in the liver, which then serves as an endocrine factor mediating GH actions in other tissues (3, 4, 5).

Evidence from both humans and animal models revealed a link between GH/IGF1 status and cancer risk (5, 6, 7, 8, 9). In particular, aberrations in the somatotropic axis have been implicated in the development of liver tumors in rodents. While GH-deficient mice are resistant to the development of carcinogen-induced liver tumors (10), transgenic mice overexpressing GH spontaneously develop hepatocellular tumors, including HCC, within a year (11, 12, 13, 14). Remarkably, liver pathology preceding the development of HCC is characterized by a sustained increase in hepatocyte turnover and development of chronic inflammation (11, 12, 15). Therefore, mice overexpressing GH constitute an appropriate model for the study of liver tumorigenesis as preneoplastic liver pathology observed in these animals recapitulates that present in patients at high risk of developing hepatic cancer (12, 16, 17).

Galectin 1 (GAL1), a proto-type member of the galectin family with affinity for β-galactoside-enriched glycoconjugates, plays key roles during liver tumorigenesis (18, 19, 20). Particularly in human HCC, GAL1 overexpression correlates with tumor cell migration and invasion, metastasis and shortened patient survival (20, 21, 22). Patients with advanced HCC had significantly higher serum levels of GAL1 compared to healthy volunteers (23). Furthermore, we have demonstrated that GAL1-overexpressing HCC cells display higher tumorigenic and metastatic potential when inoculated into immunodeficient mice (24).

Although increasing levels of GAL1 are often a hallmark of HCC aggressiveness (18, 19, 20, 21, 23, 24, 25), there is still no information on how endogenous stimuli control its expression *in vivo* and whether it plays a role in hormone-driven carcinogenesis. In this work, we show that prolonged unremitting exposure to GH induces GAL1 expression in mouse liver, which may have critical implications in tumorigenesis.

Materials and methods

Growth hormone (GH)-transgenic mice

Transgenic PEPCK-bGH mice containing the bovine *GH* gene fused to control sequences of the rat phosphoenolpyruvate carboxykinase (*Pepck*) gene have been previously described (17, 26). Hemizygous transgenic mice were produced by mating transgenic males with normal C57BL/6 X C3H F1 hybrid females purchased from the Jackson Laboratory. These mice had markedly accelerated postweaning growth, leading to a significant increase in body weight and organomegaly. Normal-sized siblings of transgenic mice were used as controls. Mice were housed five per cage in a room with controlled light (12 h light per day) and temperature (22 ± 2°C) and had free access to food (Rodent Laboratory Chow 5001; not autoclaved; 23.4% w/w protein, 4.5% w/w fat, 5.8% w/w crude fiber; LabDiet, PMI Feeds, St. Louis, MO, USA) and water.

Animals were fasted for 5 h and then killed by cervical dislocation under isofluorane anesthesia. Livers, skeletal muscles from the hind limb, hearts and kidneys were removed and stored at −70°C until use. In old GH-transgenic mice (10–13 months old), liver tumor and non-tumor areas were removed, processed in parallel and stored as described above. For histopathological analysis, a piece of liver from each animal was fixed and paraffinized following standard procedures.

Experiments were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals. Animal protocols were approved by the Southern Illinois University Laboratory Animal Care and Use Committee.

GH administration to Swiss-Webster mice

Adult (3–4 months old) Swiss-Webster female and male mice were used. Animals were housed 3–5 per cage in a room with controlled light (12 h light: 12 h darkness cycle) and temperature (22 ± 2°C). Mice had free access to a nutritionally balanced diet and tap water.

Highly purified porcine GH (Zamira Life Sciences, Knoxfield, Victoria, Australia) was subcutaneously administered to Swiss-Webster mice for 5 days under continuous infusion with an osmotic minipump (model 1007D, Alzet) delivering the hormone at a dose 1 mg/kg of body weight per day, as previously described (27). Control animals were treated in a similar fashion but saline solution was administered instead. Five days after minipump implantation, animals were fasted for 6 h and then killed by cervical dislocation. Livers were removed and stored frozen at −70°C until homogenization and preparation of liver extracts for immunoblotting (27).

The appropriateness of the experimental procedure, the required number of animals and the method of acquisition were in compliance with federal and local

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

laws and with institutional regulations. Animal protocols were approved by the Laboratory Animal Care Committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires.

Cell culture

The human HCC cell lines HepG2/C3A (ATCC CRL-10741, a clonal derivative of HepG2 cell line ATCC HB-8065) and HuH-7 (ICRB 0403) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies-Invitrogen) containing 4.5 g/L glucose, supplemented with 10% v/v fetal bovine serum (Natocor, Córdoba, Argentina), 0.002 mol/L L-glutamine and antibiotics in a humidified atmosphere of 5% CO_2 at 37°C. Experiments were performed with low-passage cell cultures (<20 passages). Cells were cultured in serum-free media for 24 h and then treated with 0.5 or 1 µg/mL human recombinant GH (European Pharmacopoeia) in the absence of serum for the indicated times. After treatment, cells were processed for quantitative RT-PCR analysis and immunoblotting.

Quantitative RT-PCR (RT-qPCR) analysis

Total RNA was extracted from liver tissue and cells using TRIzol Reagent (Invitrogen™, Thermo Fisher Scientific) following the manufacturer's instructions. cDNA was obtained using iScript cDNA synthesis kit (Bio-Rad Laboratories). For RT-qPCR, primer sets were designed for the specific amplification of galectin 1 (GAL1; encoded by the *Lgals1* gene) and Cyclophilin A or RPL13A as reference genes. Sense and antisense oligonucleotide primers were designed following general recommendations with the public software Primer BLAST and OligoPerfect™ Designer (Thermo Fisher Scientific) and were obtained from Invitrogen. The sequence of primers (5′→3′) were: *Gal1* F: TGAACCTGGGAAAAGACAGC, R: TCAGCCTGGTCAAAGGTGAT; *GAL1* F: GGAAC ATCCTCCTGGACTCA, R: ACGAAGCTCTTAGCGTCAGG; *Cyclophilin A* F: GCGTCTCCTTGAGCTGTT, R: TCAG CCTGGTCAAAGGTGAT; *RPL13A* F: TGACAAGAA AAAGCGGATGGTG, R: GCTGTCACTGCCTGGTACTT. Each sample was assayed in duplicate using 4 pmol of each primer, 1× SYBR® Select Master Mix (Applied Biosystems™, Thermo Fisher Scientific) and 25 ng of reverse-transcribed RNA in a total volume of 13 µL. Amplification was carried out in a Step One Plus™ Real-Time PCR System (Applied Biosystems™). Dissociation curves were generated at the end of each run in order to detect non-specific products and validate the results. Relative gene expression levels were calculated according to the comparative cycle threshold (Ct) method, which refers results from each experimental sample to a calibrator, in this case the average Ct value of the corresponding run, and calculates the relative change as $2^{-\Delta Ct}$. Target gene relative expression levels were normalized by Cyclophilin A expression levels. To validate this method of relative quantification, the amplification efficiencies of target and reference genes were measured and shown to be approximately equal and close to 100%.

Immunoblotting

Tissues and cells were homogenized in solubilization buffer (0.1 mol/L Hepes, pH 7.4, 1% v/v Triton X-100, 0.1 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 0.01 mol/L EDTA, 0.01 mol/L sodium vanadate, 0.002 mol/L phenylmethylsulfonyl fluoride (PMSF) and 0.035 units/mL aprotinin). Homogenates were centrifuged at 100,000***g*** at 4°C for 60 min to remove insoluble material. Protein concentration of supernatants was determined by the bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). Forty microgram total protein of each solubilized sample were resolved by 12% SDS-PAGE. For immunoblotting, proteins were transferred to PVDF membranes (Amersham Hybond P 0.45 PVDF blotting membrane, GE Healthcare Life Sciences) and probed with goat anti-GAL1 polyclonal antibody 1:1000 (sc-19277, Santa Cruz Biotechnology) overnight at 4°C, followed by a 60-min incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (sc-2020, Santa Cruz Biotechnology). Primary antibody specificity has been demonstrated in GAL1-knocked-down cells previously (28, 29). Immunoreactive bands were detected by chemiluminiscence (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific). Protein loading control could not be assessed by immunoblotting of standard proteins in the liver for GH-overexpressing mice since actin exhibits age and genotype-dependent variation in hepatic protein abundance, whereas tubulin does not react uniformly between genotypes (17, 30). Therefore, protein loading control was performed by relativizing protein content to Coomassie blue staining of PVDF membranes after blotting experiments as previously described (17, 30, 31). Of note, in experiments using cell lines and Swiss-Webster mice, protein loading control was performed by β-actin immunoblotting (rabbit anti-β-actin polyclonal antibody, 1:1000; A2066, Sigma-Aldrich). Protein quantification was performed by densitometric analysis using ImageJ software (National Institutes of Health).

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

Immunohistochemistry

Freshly dissected livers were fixed overnight in 4% w/v paraformaldehyde and embedded in paraffin. Liver sections (5 µm thickness) were transferred onto glass slides, deparaffinized and rehydrated. After antigenic recuperation with sodium citrate (pH 6.0) at 98°C for 30 min, non-specific proteins were blocked using 1% w/v BSA in PBS for 1 h followed by normal horse serum for 2 h. Liver sections were incubated overnight at 4°C with anti-GAL1 antibody (1:200, sc-19277, Santa Cruz Biotechnology). Afterward, incubation with biotin-labeled secondary antibody followed by incubation with streptavidin-horseradish peroxidase complex (R.T.U. Vectastain kit, Vector Laboratories) were each performed for 30 min at room temperature. Endogenous peroxidase activity was blocked incubating slides on PBS containing 3% v/v hydrogen peroxide for 30 min. Tissues were incubated with 1% w/v BSA in PBS instead of the primary antibody as negative control. Antigen-antibody binding was visualized with the DAB chromogen (Peroxidase substrate kit, DAB sk-4100, Vector Laboratories) and counterstaining was done 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 and images were obtained by light microscopy using a Leica DM2000 microscope with a ×40 objective, a Leica DFC400 digital camera and Leica Application Suite software (Leica Microsystems). At least 20 optical fields were examined and representative images were photographed.

Statistical analysis

GraphPad Prism statistical program by GraphPad Software was used. Results are expressed as the median ± S.E.M. of the indicated number (n) of different individuals or experiments. Two-way ANOVA and Bonferroni post-test were used to assess age and genotype, sex and genotype, or age and sex differences in GAL1 expression in normal and GH-transgenic mice. Unpaired Student's t-test was applied to compare control and transgenic mice only when old mice were analyzed. Paired Student's t-test was used to compare GAL1 expression between tumors and non-tumoral adjacent tissue. Unpaired Student's t-test was used to compare GAL1 protein levels between control and GH-treated Swiss-Webster mice for each sex. When GAL1 protein/mRNA expression was compared between control and GH-treated cells, unpaired Student's t-test, one-way or two-way ANOVA and Bonferroni post-test, were applied accordingly to the number of experimental conditions evaluated. When several groups were analyzed in parallel and/or a considerable dispersion of values was obtained for some groups, the concomitant loss of sensitivity prevented to find statistically significant differences by two-way ANOVA although these differences were evident. In these cases, unpaired Student's t-test was used to compare two groups separately. All tests were two-sided, and a P value <0.05 was considered statistically significant.

Results

GAL1 expression is upregulated in the liver of GH-transgenic mice throughout life and is evident even in the absence of neoplastic pathology

To examine the possible role of GAL1 during GH-related liver tumor progression, we first examined GAL1 expression in liver extracts of three representative ages: (a) 2-week-old, when animals of both genotypes exhibit similar body size; (b) 4-week-old, when transgenic mice start to display different body proportions than normal siblings and (c) 9-week-old (young adults), showing clear enlargement of body size and exhibiting preneoplastic changes in the liver (30). Age-matched non-transgenic siblings were used as controls and both male and female mice were analyzed in parallel.

We found elevated GAL1 protein levels in 2-week-old mice compared to older animals in both female and male transgenic mice (Fig. 1A and B). A similar profile was observed for normal mice, despite only statistically significant for males. However, when non-transgenic males and females were analyzed independently, age-differences were statistically significant for both sexes and no gender-related differences were found (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). *Gal1* mRNA expression exhibited a different age profile. In GH-transgenic mice, while similar levels between ages were obtained for females, lower mRNA levels were found in 4-week-old transgenic males in comparison to 2- and 9-week-old male mice (Fig. 1C and D). In normal mice, *Gal1* mRNA expression displayed a decrease with age, statistically significant when evaluated separately, and no differences between males and females were found, similar to what was observed for GAL1 protein levels (Supplementary Fig. 1B). Different GAL1 expression between genotypes was evident when age-matched control and GH-transgenic mice were compared. Both female and male GH-transgenic mice at 2 weeks of

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

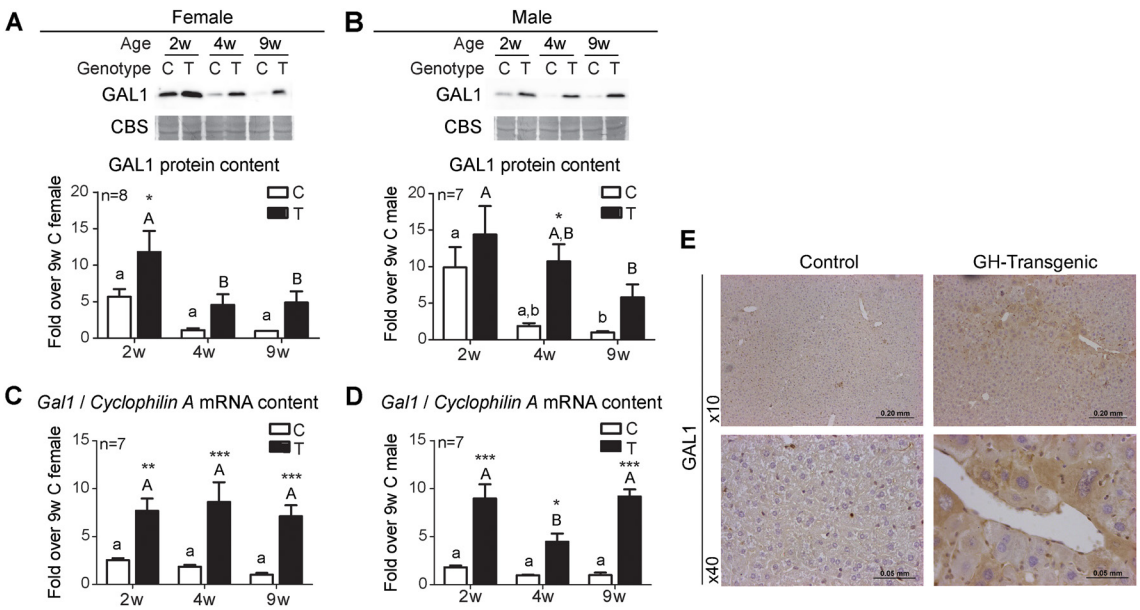

Figure 1

GAL1 expression is increased in the liver of young GH-transgenic mice. Liver extracts of control (C) and GH-transgenic (T) mice of three representative ages: 2 week-old (2 w), 4 week-old (4 w) and 9 week-old (9 w, considered young adults and used as reference) were evaluated for GAL1 protein expression by Western blotting and densitometric analysis in females and males (panels A and B, respectively). Protein loading control was performed by Coomassie blue staining (CBS) of PVDF membranes. Liver *Gal1* mRNA levels relative to *Cyclophilin A* were determined by RT-qPCR analysis in females and males (panels C and D, respectively). Data are the mean ± S.E.M. of the indicated number of samples per group (*n*), each one representing a different animal. Different letters denote significant differences by age ($P < 0.05$), small letters correspond to normal mice and capital letters to transgenic animals; asterisks indicate differences between genotypes analyzed by two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). GAL1 expression and cellular localization were evaluated by immunohistochemistry in the liver of 9-week-old animals (E). Three independent experiments were performed with reproducible results and representative microphotographs are shown for liver sections of female mice. Similar results were obtained for males (Supplementary Fig. 2A). Negative controls were performed in parallel in all sections by incubation without primary antibody (Supplementary Fig. 2B).

age showed higher GAL1 protein content in the liver than control mice, but this increase was statistically different only in females (Fig. 1A and B). GAL1 protein levels in the liver of 4- and 9-week-old transgenic mice were also upregulated with respect to age-matched control mice (Fig. 1A and B). These differences were statistically significant when each age was analyzed separately by Student's *t*-test ($P<0.05$). *Gal1* mRNA expression was also higher in the liver of transgenic animals as compared with control mice at every age studied and for both sexes (Fig. 1C and D).

To further confirm GAL1 expression and assess its cellular localization, immunohistochemical studies were performed in liver sections from 9-week-old transgenic and control mice. Representative microphotographs are shown in Fig. 1E for female mice; yet, similar results were obtained for males (Supplementary Fig. 2A). GAL1 staining was stronger in liver sections from transgenic mice, particularly at the cytoplasm of enlarged hepatocytes around the vasculature structures. Areas of dysplasia with large cellular and nuclear size and nuclear polymorphism of hepatocytes, predominantly in the centrolobullar areas within the liver of young adult transgenic mice were previously reported (17). Negative controls demonstrated the specificity of the reaction (Supplementary Fig. 2B).

Thus, prolonged exposure to high levels of GH upregulates GAL1 expression in the liver of GH-transgenic mice at early ages, when animals display no or minimal histological alterations in this tissue, and also in young adults with preneoplastic liver pathology.

GAL1 upregulation in the liver of GH-transgenic mice is sustained during aging and particularly enhanced in hepatic tumors

Upregulation of GAL1 in human HCC correlates with tumor progression and aggressiveness (20, 22). GH-transgenic mice develop spontaneous hepatocellular tumors, including adenoma and carcinoma within 1 year of age (11, 12). Therefore, we determined GAL1 expression in the liver of 10–13-month-old GH-transgenic mice. These mice are regarded as old since GH overexpression is

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

related to premature aging and a shorter mean life span than normal mice (13, 14). GAL1 protein and mRNA levels significantly increased in the liver of these animals as compared to age-matched control mice (Fig. 2A and B). The protein levels of GAL1 in males and in females were analyzed separately. In females, GAL1 protein content was approximately 28-fold higher in GH-transgenic mice than in normal controls, while in males GH-transgenic animals displayed a 6.6-fold increase compared with controls (Fig. 2A). In the case of *Gal1* mRNA content, both sexes were analyzed in parallel. In control mice, *Gal1* mRNA levels were lower in males than in females (statistically not significant by two-way ANOVA, significantly different by Student's *t*-test, $P<0.001$). In contrast, in transgenic animals, these levels were slightly higher in males than in females; however, the differences observed in *Gal1* mRNA content between sexes did not achieve statistical significance. When we analyzed differences between genotypes, we observed that *Gal1* mRNA levels were higher in old GH-transgenic mice than in their respective normal controls in both sexes. *Gal1* mRNA content increased 4.5-fold in female GH-transgenic mice and 15-fold in males, compared to normal controls of the same sex (Fig. 2B).

Remarkably, we found that liver tumors of old transgenic mice showed increased GAL1 protein expression compared to non-tumoral adjacent tissues, both in females and males (Fig. 2C). *Gal1* mRNA levels also increased in hepatic tumors, but this increase was statistically significant only in females (Fig. 2D). In immunohistochemical assays, GAL1 staining in old transgenic mice was observed in both tumoral and non-tumoral liver tissues, but the strongest intensity

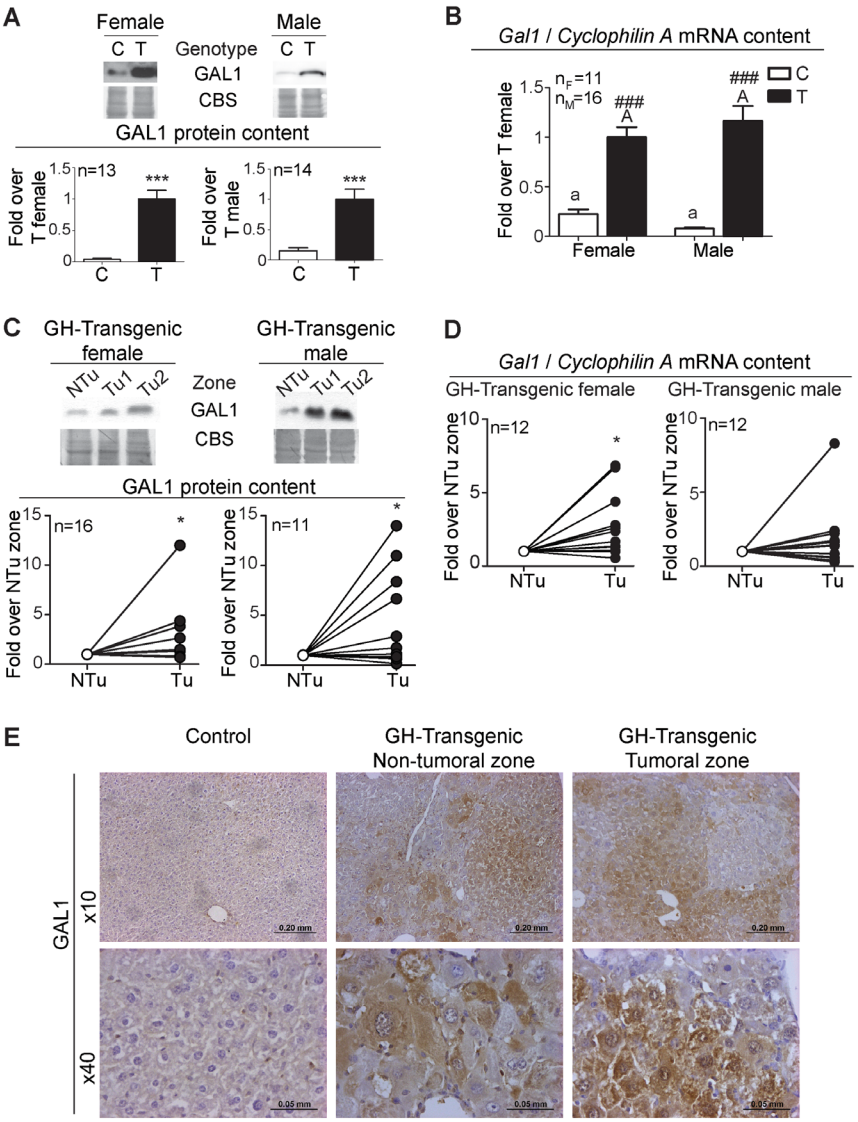

Figure 2
GAL1 is upregulated in the liver and in hepatic tumors of old GH-transgenic mice. GAL1 expression was assessed in the liver of control (C) and GH-transgenic (T) mice of 10–13 months old by Western blotting and densitometric analysis (A) and RT-qPCR assay (B). Protein loading control was performed by Coomassie blue staining (CBS) of PVDF membranes. Tumoral (Tu) and non-tumoral (NTu) zones for the same GH-transgenic animal were processed in parallel and GAL1 protein content (C) and mRNA levels relative to *Cyclophilin A* (D) were determined. Data are the mean ± S.E.M. of the indicated number of samples per group (*n*), each one representing a different animal. The same letter denotes no significant difference between sexes, small letters correspond to normal mice and capital letters to transgenic animals; the symbol # indicates differences between genotypes analyzed by two-way ANOVA followed by Bonferroni post-test ($^{\#\#\#}P<0.001$). Asterisks indicate differences analyzed by Student's *t*-test ($^{*}P<0.05$, $^{***}P<0.001$). Liver sections from control and transgenic mice were processed for immunohistochemistry to analyze GAL1 expression and localization. Tumoral (Tu) and non-tumoral (NTu) tissues were processed in parallel (E). Three independent experiments were performed with reproducible results and representative microphotographs are shown for liver sections of female mice. Similar results were obtained for males (data not shown). Negative controls were performed by omitting primary antibody (Supplementary Fig. 2C).

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

was particularly evident within tumors (Fig. 2E and Supplementary Fig. 2C). Similar to 9-week-old animals, GAL1 staining was detected mainly at the cytoplasm of enlarged hepatocytes around blood vessels.

Therefore, GAL1 upregulation in the liver of GH-transgenic mice was preserved until advanced ages and was particularly high in hepatic tumors, suggesting the possible role of this lectin at different stages of hepatocarcinogenesis.

GAL1 is not upregulated in other tissues of GH-transgenic mice

To study the influence of prolonged exposure to high GH levels in other tissues of transgenic mice, we assessed GAL1 expression in kidney, heart and skeletal muscle extracts from adult females. Immunoblotting experiments revealed that GAL1 upregulation was not a general outcome of GH overexpression in every GH-target tissue, since GAL1 levels were not increased in kidney, heart or skeletal muscle of GH-transgenic mice (Fig. 3).

Continuous administration of GH increases GAL1 expression in mouse liver

To confirm our findings, we then investigated if GAL1 upregulation was also induced upon continuous exposure to GH in another experimental model. GH was subcutaneously delivered to young adult Swiss-Webster mice by continuous infusion with osmotic minipumps for 5 days. Notably, sustained administration of GH also increased liver GAL1 protein expression in males, but not in females (Fig. 4). The sexual dimorphism observed is in line with a previous report describing that continuous administration of the hormone for 5 days caused a decrease on the expression of major urinary proteins (MUPs) in the liver in male mice, but it had no effect in female mice (27). Other authors also showed sexual differences in the liver expression of several genes and upon continuous administration of GH (32, 33, 34). Thus, GH may induce GAL1 upregulation in mouse liver not only when overexpressed as a transgene throughout life, but also when this hormone is continuously administered for a short period.

GH induces GAL1 expression in human HCC cells

To investigate whether the upregulation of GAL1 observed in mouse liver is a direct consequence of GH, we then evaluated its effect on GAL1 expression in human HepG2 and HuH-7 HCC cells. We previously demonstrated that the upregulation of GAL1 promotes HepG2 cell proliferation and epithelial-mesenchymal transition *in vitro*, and tumor growth and metastasis *in vivo* (24, 28, 29). We first evaluated GAL1 protein expression in HepG2 cells treated with GH for different times in parallel, and found that at 30 min GH treatment was associated with higher mean levels of GAL1, which did not attain statistical significance since the number of experiments of these pilot experiments was low ($n=4$) (Supplementary Fig. 3A). Moreover, GH appeared to exert a rapid and transient effect, since no apparent changes were observed upon hormone treatment for 24 h (Supplementary Fig. 3B). As we observed similar effects on GAL1 protein levels using both supraphysiological GH concentrations of 0.5 and 1 µg/mL in HepG2 cells, we continued performing the experiments with the higher one, in order to mimic the increased levels of GH in transgenic animals (Supplementary Fig. 3C). We therefore performed independent experiments to determine GAL1 protein content in cells incubated in the presence of GH for 30 min, both in HepG2 and in HuH-7 cells, and confirmed that this treatment significantly increased GAL1 protein content in both cell lines (Fig. 5A).

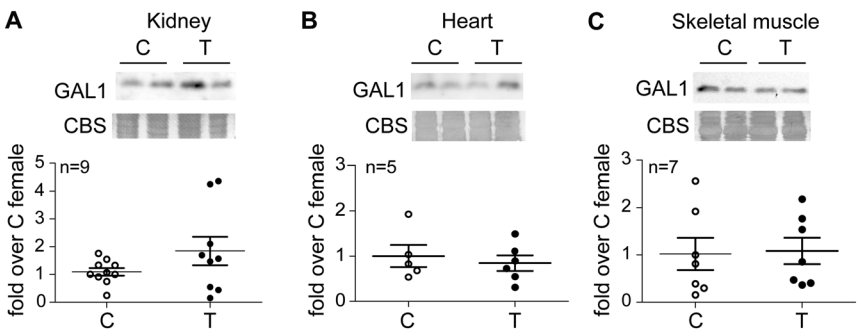

Figure 3
Prolonged exposure to GH does not increase GAL1 expression in the kidney, muscle or heart of GH-transgenic mice. Kidney (A), heart (B) and skeletal muscle (C) extracts from adult control (C) and GH-transgenic (T) females were evaluated for GAL1 expression by Western blotting and analyzed by densitometry. Protein loading control was performed by Coomassie blue staining (CBS) of PVDF membranes. Data are the mean ± S.E.M. of the indicated number of samples per group (*n*), each one representing a different animal. No significant differences were obtained when analyzed by Student's *t* test.

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

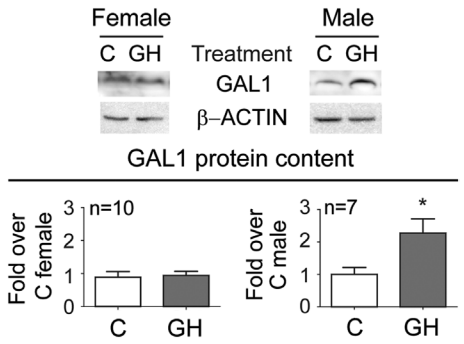

Figure 4
GAL1 expression is induced in the liver of GH-treated Swiss-Webster mice. Liver extracts of female and male mice treated for 5 days with GH (1 mg/kg of body weight per day) by continuous infusion with an osmotic minipump (GH) or their respective controls (C) were evaluated for GAL1 protein expression by Western blotting and analyzed by densitometry. Protein loading control was performed by β-actin immunoblotting. Data are the mean ± S.E.M. of the indicated number of samples per group (*n*), each one representing a different animal. Asterisks indicate differences induced by GH stimulus analyzed by Student's *t*-test (*$P < 0.05$).

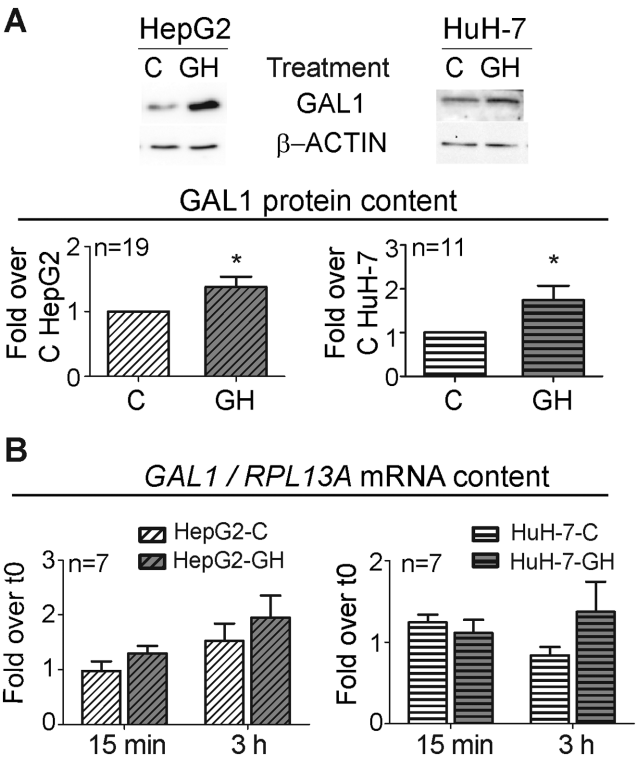

Figure 5
GAL1 expression is induced by GH in human HCC cells *in vitro*. Western blotting and densitometric analysis showing GAL1 protein expression in HepG2 and HuH-7 HCC cells treated with serum-free media containing GH (1 µg/ml) or vehicle (C, control cells used as reference) for 30 min (A). Protein loading control was performed by β-actin immunoblotting. *GAL1* mRNA levels relative to *RPL13A* were determined by RT-qPCR analysis in cells cultured in the presence of GH (1 µg/ml) (HepG2-GH and HuH-7-GH) and in control cells (HepG2-C and HuH-7-C) for 15 min or 3 h (B). Results are expressed as fold change versus values measured for untreated cells at the beginning of the experiment (t0). Data are the mean ± S.E.M. of the indicated number of different experiments (*n*). Asterisks indicate differences induced by GH-stimulus analyzed by Student's *t*-test (*$P < 0.05$) (A). No significant differences were found by two-way ANOVA followed by Bonferroni post-test analysis (B).

The effect of GH treatment on *GAL1* mRNA levels was less clear, with a tendency towards an increase after 3 h in both cell lines, and at 15 min only in HepG2 cells (Fig. 5B); however, these differences were not significantly different when analyzed by two-way ANOVA.

Therefore, GH upregulates GAL1 protein levels *in vitro* in human HCC cells. Although the precise mechanism is far from being elucidated, these results indicate that GH may act directly on hepatocytes to promote GAL1 protein expression.

Discussion

Accumulating evidence suggests a remarkable contribution of GAL1 to HCC progression, aggressiveness and metastasis (20, 22). Because increased GH levels are associated with poor patient survival in human HCC (35), we investigated the involvement of GAL1 in GH-related liver tumor progression using transgenic mice overexpressing this hormone. Our findings demonstrate that both GAL1 protein and mRNA expression were elevated in the liver of 2-week-old control mice, suggesting upregulation of this lectin early during the postnatal period. Subsequently, liver GAL1 expression declined considerably. These results are in line with previous findings showing GAL1 upregulation during liver regeneration after partial hepatectomy (36), whereas low levels of this protein are found in human normal adult liver (18, 19).

Remarkably, we found that prolonged exposure to GH *in vivo* upregulated GAL1 in the liver of transgenic animals and this effect was accentuated in tumors of older mice. Supporting these findings, GH induced hepatic GAL1 expression when this hormone was continuously administered for a short period. Interestingly, the results obtained with murine HCC models suggested that GAL1 may act as a protective anti-inflammatory agent at early stages of chronic liver pathology and as a pro-tumorigenic agent at late stages of the disease contributing to HCC growth and metastasis (37, 38). Because of the broad immunosuppressive and pro-angiogenic activities of GAL1 within tumor microenvironments (20, 29, 39), its upregulation could, at least in part, mediate GH-driven hepatocarcinogenesis. Recently, it was demonstrated that mRNA and protein levels of galectin 3, another member of the galectin family, are increased in a murine ischemia-reperfusion model flap.

https://ec.bioscientifica.com
https://doi.org/10.1530/EC-19-0292

Notably, GH administration ameliorated the inflammatory response, apoptosis and the pathological damage of the ischemia-reperfusion flap and also, partially reduced galectin 3 protein levels (40). These results suggest that within an inflammatory context, not only in the liver, GH may modulate the expression of galectins.

We have previously demonstrated that several signaling pathways involved in cell growth, proliferation and survival are deregulated in preneoplastic livers of GH-transgenic mice, and these molecular alterations resemble those found in many human tumors (17, 41). Interestingly, we also reported that many signaling mediators and effectors of these oncogenic pathways were also induced by GAL1 overexpression in human HCC cells and that this lectin contributes to tumor growth and dissemination (24, 28, 29). Here, we report that GH rapidly induces GAL1 protein expression in human HCC cells, suggesting that this lectin could be implicated in hormone-driven liver carcinogenesis.

Increased mRNA and protein levels of both GAL1 and GH were observed in human HCC compared with non-tumoral liver tissue (18, 19, 35). Furthermore, GAL1 and GH overexpression or their elevated serum levels correlated with advanced HCC, metastasis and worse clinical outcome (21, 23, 25, 35). Although the precise mechanisms underlying GH-induced GAL1 expression are yet to be elucidated, our findings suggest a potential role for this lectin as a mediator of GH-driven tumorigenesis and potential biomarker of liver cancer risk that should be further explored.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0292.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Universidad de Buenos Aires (grant numbers 20020120200122BA to J G M, 20020150100005BA to M F T, 20020130100208BA to A I S); the Consejo Nacional de Investigaciones Científicas y Técnicas (grant number 11220150100647 to M F T); the Agencia Nacional de Promoción Científica y Tecnológica (grant numbers PICT-2015-1100 to J G M, PICT-2016-1139 to M F T) and the National Institute on Aging of the National Institutes of Health (grant number R01AG019899 to A B).

Acknowledgements

The authors thank Samuel A McFadden (Southern Illinois University School of Medicine) for his laboratory assistance.

References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA & Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* 2018 **68** 394–424. (https://doi.org/10.3322/caac.21492)

2 Park JW, Chen M, Colombo M, Roberts LR, Schwartz M, Chen PJ, Kudo M, Johnson P, Wagner S, Orsini LS, *et al*. Global patterns of hepatocellular carcinoma management from diagnosis to death: the BRIDGE Study. *Liver International* 2015 **35** 2155–2166. (https://doi.org/10.1111/liv.12818)

3 Chia DJ. Minireview: mechanisms of growth hormone-mediated gene regulation. *Molecular Endocrinology* 2014 **28** 1012–1025. (https://doi.org/10.1210/me.2014-1099)

4 Blum WF, Alherbish A, Alsagheir A, El Awwa A, Kaplan W, Koledova E & Savage MO. The growth hormone-insulin-like growth factor-I axis in the diagnosis and treatment of growth disorders. *Endocrine Connections* 2018 **7** R212–R222. (https://doi.org/10.1530/EC-18-0099)

5 Guevara-Aguirre J, Guevara A, Palacios I, Pérez M, Prócel P & Terán E. GH and GHR signaling in human disease. *Growth Hormone and IGF Research* 2018 **38** 34–38. (https://doi.org/10.1016/j.ghir.2017.12.006)

6 Jenkins PJ, Mukherjee A & Shalet SM. Does growth hormone cause cancer? *Clinical Endocrinology* 2006 **64** 115–121. (https://doi.org/10.1111/j.1365-2265.2005.02404.x)

7 Perry JK, Liu DX, Wu ZS, Zhu T & Lobie PE. Growth hormone and cancer: an update on progress. *Current Opinion in Endocrinology, Diabetes, and Obesity* 2013 **20** 307–313. (https://doi.org/10.1097/MED.0b013e328363183a)

8 Lapkina-Gendler L, Rotem I, Pasmanik-Chor M, Gurwitz D, Sarfstein R, Laron Z & Werner H. Identification of signaling pathways associated with cancer protection in Laron syndrome. *Endocrine-Related Cancer* 2016 **23** 399–410. (https://doi.org/10.1530/ERC-16-0054)

9 Terzolo M, Reimondo G, Berchialla P, Ferrante E, Malchiodi E, Marinis LD, Pivonello R, Grottoli S, Losa M, Cannavo S, *et al*. Acromegaly is associated with increased cancer risk: a survey in Italy. *Endocrine-Related Cancer* 2017 **24** 495–504. (https://doi.org/10.1530/ERC-16-0553)

10 Bugni JM, Poole TM & Drinkwater NR. The little mutation suppresses DEN-induced hepatocarcinogenesis in mice and abrogates genetic and hormonal modulation of susceptibility. *Carcinogenesis* 2001 **22** 1853–1862. (https://doi.org/10.1093/carcin/22.11.1853)

11 Orian JM, Tamakoshi K, Mackay IR & Brandon MR. New murine model for hepatocellular carcinoma: transgenic mice expressing metallothionein-ovine growth hormone fusion gene. *Journal of the National Cancer Institute* 1990 **82** 393–398. (https://doi.org/10.1093/jnci/82.5.393)

12 Snibson KJ, Bhathal PS, Hardy CL, Brandon MR & Adams TE. High, persistent hepatocellular proliferation and apoptosis precede hepatocarcinogenesis in growth hormone transgenic mice. *Liver* 1999 **19** 242–252. (https://doi.org/10.1111/j.1478-3231.1999.tb00042.x)

13 Bartke A. Can growth hormone (GH) accelerate aging? Evidence from GH-transgenic mice. *Neuroendocrinology* 2003 **78** 210–216. (https://doi.org/10.1159/000073704)

14 Kopchick JJ, List EO, Kelder B, Gosney ES & Berryman DE. Evaluation of growth hormone (GH) action in mice: discovery of GH receptor antagonists and clinical indications. *Molecular and Cellular Endocrinology* 2014 **386** 34–45. (https://doi.org/10.1016/j.mce.2013.09.004)

15 Quaife CJ, Mathews LS, Pinkert CA, Hammer RE, Brinster RL & Palmiter RD. Histopathology associated with elevated levels of growth hormone and insulin-like growth factor I in transgenic mice. *Endocrinology* 1989 **124** 40–48. (https://doi.org/10.1210/endo-124-1-40)

16 Snibson KJ. Hepatocellular kinetics and the expression of growth hormone (GH) in the livers and liver tumours of GH-transgenic mice. *Tissue and Cell* 2002 **34** 88–97. (https://doi.org/10.1016/S0040-8166(02)00012-5)

17 Miquet JG, Freund T, Martinez CS, González L, Díaz ME, Micucci GP, Zotta E, Boparai RK, Bartke A, Turyn D, *et al*. Hepatocellular

alterations and dysregulation of oncogenic pathways in the liver of transgenic mice overexpressing growth hormone. *Cell Cycle* 2013 **12** 1042–1057. (https://doi.org/10.4161/cc.24026)
18 Chung EJ, Sung YK, Farooq M, Kim Y, Im S, Tak WY, Hwang YJ, Kim YI, Han HS, Kim JC, *et al*. Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray. *Molecules and Cells* 2002 **14** 382–387.
19 Kondoh N, Hada A, Ryo A, Shuda M, Arai M, Matsubara O, Kimura F, Wakatsuki T & Yamamoto M. Activation of galectin-1 gene in human hepatocellular carcinoma involves methylation-sensitive complex formations at the transcriptional upstream and downstream elements. *International Journal of Oncology* 2003 **23** 1575–1583. (https://doi.org/10.3892/ijo.23.6.1575)
20 Bacigalupo ML, Manzi M, Rabinovich GA & Troncoso MF. Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation of hepatocellular carcinoma. *World Journal of Gastroenterology* 2013 **19** 8831–8849. (https://doi.org/10.3748/wjg.v19.i47.8831)
21 Spano D, Russo R, Di Maso V, Rosso N, Terracciano LM, Roncalli M, Tornillo L, Capasso M, Tiribelli C & Iolascon A. Galectin-1 and its involvement in hepatocellular carcinoma aggressiveness. *Molecular Medicine* 2010 **16** 102–115. (https://doi.org/10.2119/molmed.2009.00119)
22 Bacigalupo ML, Carabias P & Troncoso MF. Contribution of galectin-1, a glycan-binding protein, to gastrointestinal tumor progression. *World Journal of Gastroenterology* 2017 **23** 5266–5281. (https://doi.org/10.3748/wjg.v23.i29.5266)
23 Yeh CC, Hsu CH, Shao YY, Ho WC, Tsai MH, Feng WC & Chow LP. Integrated Stable Isotope Labeling by amino Acids in Cell Culture (SILAC) and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) quantitative proteomic analysis identifies galectin-1 as a potential biomarker for predicting sorafenib resistance in liver cancer. *Molecular and Cellular Proteomics* 2015 **14** 1527–1545. (https://doi.org/10.1074/mcp.M114.046417)
24 Espelt MV, Croci DO, Bacigalupo ML, Carabias P, Manzi M, Elola MT, Muñoz MC, Dominici FP, Wolfenstein-Todel C, Rabinovich GA, *et al*. Novel roles of galectin-1 in hepatocellular carcinoma cell adhesion, polarization, and in vivo tumor growth. *Hepatology* 2011 **53** 2097–2106. (https://doi.org/10.1002/hep.24294)
25 Wu H, Chen P, Liao R, Li YW, Yi Y, Wang JX, Sun TW, Zhou J, Shi YH, Yang XR, *et al*. Overexpression of galectin-1 is associated with poor prognosis in human hepatocellular carcinoma following resection. *Journal of Gastroenterology and Hepatology* 2012 **27** 1312–1319. (https://doi.org/10.1111/j.1440-1746.2012.07130.x)
26 McGrane MM, de Vente J, Yun J, Bloom J, Park E, Wynshaw-Boris A, Wagner T, Rottman FM & Hanson RW. Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic mice. *Journal of Biological Chemistry* 1988 **263** 11443–11451.
27 Díaz ME, Miquet JG, Rossi SP, Irene PE, Sotelo AI, Frungieri MB, Turyn D & González L. GH administration patterns differently regulate epidermal growth factor signaling. *Journal of Endocrinology* 2014 **221** 309–323. (https://doi.org/10.1530/JOE-13-0447)
28 Bacigalupo ML, Manzi M, Espelt MV, Gentilini LD, Compagno D, Laderach DJ, Wolfenstein-Todel C, Rabinovich GA & Troncoso MF. Galectin-1 triggers epithelial-mesenchymal transition in human hepatocellular carcinoma cells. *Journal of Cellular Physiology* 2015 **230** 1298–1309. (https://doi.org/10.1002/jcp.24865)
29 Manzi M, Bacigalupo ML, Carabias P, Elola MT, Wolfenstein-Todel C, Rabinovich GA, Espelt MV & Troncoso MF. Galectin-1 controls the proliferation and migration of liver sinusoidal endothelial cells and their interaction with hepatocarcinoma cells. *Journal of Cellular Physiology* 2016 **231** 1522–1533. (https://doi.org/10.1002/jcp.25244)
30 Martinez CS, Piazza VG, Díaz ME, Boparai RK, Arum O, Ramírez MC, González L, Becú-Villalobos D, Bartke A, Turyn D, *et al*. GH/STAT5 signaling during the growth period in livers of mice overexpressing GH. *Journal of Molecular Endocrinology* 2015 **54** 171–184. (https://doi.org/10.1530/JME-14-0262)
31 Welinder C & Ekblad L. Coomassie staining as loading control in Western blot analysis. *Journal of Proteome Research* 2011 **10** 1416–1419. (https://doi.org/10.1021/pr1011476)
32 Li J, Wan Y, Na S, Liu X, Dong G, Yang Z, Yang J & Yue J. Sex-dependent regulation of hepatic CYP3A by growth hormone: roles of HNF6, C/EBPα, and RXRα. *Biochemical Pharmacology* 2015 **93** 92–103. (https://doi.org/10.1016/j.bcp.2014.10.010)
33 Lau-Corona D, Suvorov A & Waxman DJ. Feminization of male mouse liver by persistent growth hormone stimulation: activation of sex-biased transcriptional networks and dynamic changes in chromatin states. *Molecular and Cellular Biology* 2017 **37** e00301-17. (https://doi.org/10.1128/MCB.00301-17)
34 Waxman DJ & O'Connor C. Growth hormone regulation of sex-dependent liver gene expression. *Molecular Endocrinology* 2006 **20** 2613–2629. (https://doi.org/10.1210/me.2006-0007)
35 Kong X, Wu W, Yuan Y, Pandey V, Wu Z, Lu X, Zhang W, Chen Y, Wu M, Zhang M, *et al*. Human growth hormone and human prolactin function as autocrine/paracrine promoters of progression of hepatocellular carcinoma. *Oncotarget* 2016 **7** 29465–29479. (https://doi.org/10.18632/oncotarget.8781)
36 Potikha T, Ella E, Cerliani JP, Mizrahi L, Pappo O, Rabinovich GA, Galun E & Goldenberg DS. Galectin-1 is essential for efficient liver regeneration following hepatectomy. *Oncotarget* 2016 **7** 31738–31754. (https://doi.org/10.18632/oncotarget.9194)
37 Potikha T, Stoyanov E, Pappo O, Frolov A, Mizrahi L, Olam D, Shnitzer-Perlman T, Weiss I, Barashi N, Peled A, *et al*. Interstrain differences in chronic hepatitis and tumor development in a murine model of inflammation-mediated hepatocarcinogenesis. *Hepatology* 2013 **58** 192–204. (https://doi.org/10.1002/hep.26335)
38 Potikha T, Pappo O, Mizrahi L, Olam D, Maller SM, Rabinovich GA, Galun E & Goldenberg DS. Lack of galectin-1 exacerbates chronic hepatitis, liver fibrosis, and carcinogenesis in murine hepatocellular carcinoma model. *FASEB Journal* 2019 **33** 7995–8007. (https://doi.org/10.1096/fj.201900017R)
39 Croci DO, Salatino M, Rubinstein N, Cerliani JP, Cavallin LE, Leung HJ, Ouyang J, Ilarregui JM, Toscano MA, Domaica CI, *et al*. Disrupting galectin-1 interactions with N-glycans suppresses hypoxia-driven angiogenesis and tumorigenesis in Kaposi's sarcoma. *Journal of Experimental Medicine* 2012 **209** 1985–2000. (https://doi.org/10.1084/jem.20111665)
40 Liu B, Xu Q, Wang J, Lin J, Pei Y, Cui Y, Wang G & Zhu L. Recombinant human growth hormone treatment of mice suppresses inflammation and apoptosis caused by skin flap ischemia-reperfusion injury. *Journal of Cellular Biochemistry* 2019 [epub]. (https://doi.org/10.1002/jcb.29122)
41 Miquet JG, González L, Matos MN, Hansen CE, Louis A, Bartke A, Turyn D & Sotelo AI. Transgenic mice overexpressing GH exhibit hepatic upregulation of GH-signaling mediators involved in cell proliferation. *Journal of Endocrinology* 2008 **198** 317–330. (https://doi.org/10.1677/JOE-08-0002)

Received in final form 12 June 2019
Accepted 4 July 2019
Accepted Preprint published online 4 July 2019