Published in final edited form as:

Hepatology. 2015 February ; 61(2): 613–626. doi:10.1002/hep.27408.

Growth hormone resistance exacerbates cholestasis-induced murine liver fibrosis

Patricia Stiedl¹, Robert McMahon², Leander Blaas³, Victoria Stanek¹, Jasmin Svinka⁴, Beatrice Grabner¹, Gernot Zollner⁵, Sonja M. Kessler^{6,7}, Thierry Claudel², Mathias Müller⁸, Wolfgang Mikulits⁴, Martin Bilban⁹, Harald Esterbauer⁹, Robert Eferl⁴, Johannes Haybaeck⁶, Michael Trauner², and Emilio Casanova^{1,10}

¹Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria

²Hans Popper Laboratory of Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria

³Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, Sweden

⁴Department of Internal Medicine I, Comprehensive Cancer Center CCC, Institute for Cancer Research, Medical University of Vienna, Vienna, Austria

⁵Division of Gastroenterology and Hepatology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

⁶Institute of Pathology, Medical University of Graz, Graz, Austria

⁷Pharmaceutical Biology, Department of Pharmacy, Saarland University, Saarbrücken, Germany

⁸Biomodels Austria, Institute of Animal Breeding and Genetics, University of Veterinary Medicine Vienna, Vienna, Austria

⁹Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

¹⁰Institute of Pharmacology, Center of Physiology and Pharmacology, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria

Abstract

Growth hormone (GH) resistance has been associated with liver cirrhosis in humans but its contribution to the disease remains controversial. In order to elucidate whether GH resistance plays a causal role in the establishment and development of liver fibrosis, or rather represents a major consequence thereof, we challenged mice lacking the Growth hormone receptor gene ($Ghr^{-/-}$, a model for GH resistance) by crossing them with $Mdr2$ knockout mice ($Mdr2^{-/-}$), a mouse model of inflammatory cholestasis and liver fibrosis.

$Ghr^{-/-};Mdr2^{-/-}$ mice showed elevated serum markers associated with liver damage and cholestasis, extensive bile duct proliferation and increased collagen deposition relative to $Mdr2^{-/-}$ mice, thus suggesting a more severe liver fibrosis phenotype. Additionally, $Ghr^{-/-};Mdr2^{-/-}$ mice had a pronounced down-regulation of hepato-protective genes *Hnf6*, *Egfr* and *Igf-1*, and significantly

increased levels of ROS and apoptosis in hepatocytes, compared to control mice. Moreover, single knockout mice (*Ghr*^{-/-}) fed with a diet containing 1% cholic acid displayed an increase in hepatocyte ROS production, hepatocyte apoptosis and bile infarcts compared to their wildtype littermates, indicating that loss of *Ghr* renders hepatocytes more susceptible to toxic bile acid accumulation. Surprisingly, and despite their severe fibrotic phenotype, *Ghr*^{-/-};*Mdr2*^{-/-} mice displayed a significant decrease in tumour incidence compared to *Mdr2*^{-/-} mice, indicating that loss of *Ghr* signaling may slow the progression from fibrosis/cirrhosis to cancer in the liver.

Conclusion—Our findings suggest that GH resistance dramatically exacerbates liver fibrosis in a mouse model of inflammatory cholestasis, therefore suggesting that GH resistance plays a causal role in the disease and provides a novel target for the development of liver fibrosis treatments.

Keywords

ROS; IGF-1; cancer; apoptosis; bile acids

Introduction

Liver fibrosis is the pathological accumulation of fibrous scar tissue in the liver, eventually leading to a loss of hepatic function.¹ Prolonged insults, such as chronic infections (hepatitis), toxins, alcohol abuse, morbid obesity, nonalcoholic fatty liver disease and cholestasis can lead to the development of this severe phenotype.² The disease is often asymptomatic and can progress to liver cirrhosis and may ultimately lead to hepatocellular carcinoma (HCC). Liver damage can result in apoptosis and necrosis of hepatocytes which subsequently leads to the activation of Kupffer cells. These liver resident macrophages produce, amongst other cytokines, transforming growth factor beta (TGF-β), monocyte chemoattractant protein-1 (MCP-1) and platelet-derived growth factor (PDGF), which activate and mobilise quiescent hepatic stellate cells (HSCs). Once activated, HSCs differentiate into fibrogenic myofibroblasts and secrete TGF-β, Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), which then act in an autocrine and paracrine manner promoting liver remodeling by secretion of various extracellular matrix (ECM) components and ECM remodeling enzymes which ultimately leads to liver fibrosis.^{1–3}

Growth hormone (GH) signaling plays a central role in the physiology and pathophysiology of the liver. Binding of GH to its cognate receptor (Growth hormone receptor, GHR) causes receptor dimerization and Janus kinase 2 (JAK2) activation. Activated JAK2 phosphorylates signal transducer and activator of transcription 5 (STAT5) which, upon dimerization, translocates to the nucleus where it acts as a transcription factor controlling the expression of various genes responsible for vital liver function.⁴ Growth hormone resistance [a condition defined by high levels of circulating GH and low levels of insulin-like growth factor 1 (IGF-1)] is commonly found in patients suffering from liver fibrosis and cirrhosis.⁵ It has been extensively debated, whether the alterations of the GH-IGF-1 axis observed in liver cirrhosis are mere consequences of the malfunctioning liver in advanced stages of the disease or, whether they promote the onset and progression of liver fibrosis.⁵ To address this question, we challenged GHR knockout mice (*Ghr*^{-/-}, a mouse model for GH resistance⁶), by crossing them with the Multidrug resistance gene 2 knockout mouse (*Mdr2* (*Abcb4*)^{-/-}), a mouse model of inflammatory cholestasis and liver fibrosis.⁷

Materials and Methods

Animals

Mice carrying the *Ghr/bp* deletion ($Ghr^{-/-}$) and $Mdr2^{-/-}$ mice were described previously^{6,7}. By respective intercrossing we generated double knockout mice ($Ghr^{-/-};Mdr2^{-/-}$) and maintained animals on a mixed genetic background (129Sv /C57BL/6). $Mdr2^{+/-}$ mice were disease-free and used as littermate controls. For experimental procedures we used 8 week old male mice unless otherwise stated. Bile acid overload was induced in mice with a diet containing 1% cholic acid for 8 weeks. Mice were kept at the Decentralized Biomedical Facilities, Medical University of Vienna, under standardized conditions, and all animal experiments were carried out according to an ethical animal license protocol and contract approved by the Medical University of Vienna and Austrian Federal Ministry of Education, Arts and Culture authorities.

Additional Materials and Methods

Animal and histology procedures, quantitative reverse transcription polymerase chain reaction (qRT-PCR), serum biochemistry, Western Blot analysis, hydroxyproline measurements, microarray analysis, immunohistochemistry, primary hepatocyte isolation and cell treatments and statistical analysis are described in the Supporting Materials and Methods.

Results

$Ghr^{-/-};Mdr2^{-/-}$ mice develop hepatic injury and impaired bile acid homeostasis

Macroscopic analysis revealed that livers of $Ghr^{-/-};Mdr2^{-/-}$ mice were smaller than littermate controls (Wt, $Mdr2^{-/-}$ and $Ghr^{-/-}$) and displayed enlarged gall bladders at 8 weeks of age (Fig. 1A). $Mdr2^{-/-}$ mice and $Ghr^{-/-};Mdr2^{-/-}$ mice showed a significant increase in liver weight/body weight ratio, in contrast $Ghr^{-/-}$ mice showed a significant decrease compared to Wt littermates (Fig. 1B). As expected, $Ghr^{-/-}$ and $Ghr^{-/-};Mdr2^{-/-}$ mice show increased levels of circulating GH and low serum IGF-1 levels (Fig. 1C). Furthermore, mRNA expression of *Als* was down-regulated in $Ghr^{-/-}$ and $Ghr^{-/-};Mdr2^{-/-}$ mice, while *Igf1bp1* mRNA expression was up-regulated (Supporting Fig. 1E), thus further reducing IGF-1 bioavailability.⁵ These results are in line with a severe GH resistance phenotype in these mice. Serum parameter levels indicative of liver injury, such as alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine aminotransferase (ALT), were strongly increased in $Ghr^{-/-};Mdr2^{-/-}$ mice compared to all control mice (Fig. 1D). Furthermore, circulating levels of bilirubin and bile acids levels were greatly elevated in sera obtained from $Ghr^{-/-};Mdr2^{-/-}$ animals, suggesting a severe cholestatic phenotype (Fig. 1D). Expression analysis of genes implicated in bile acid metabolism revealed a down-regulation of the basolateral bile acid importer organic anion transporter polypeptide 1 (*Oatp1*) in $Ghr^{-/-}$ and $Ghr^{-/-};Mdr2^{-/-}$ mice (Supporting Fig. 1D). Interestingly, we did not observe *Cyp7A1* repression in $Ghr^{-/-};Mdr2^{-/-}$ mice, despite the high levels of bile acids in these animals (Supporting Fig. 1D). These results propose that $Ghr^{-/-};Mdr2^{-/-}$ mice display severe liver injury, strong cholestasis and deregulation of bile acid homeostasis.

Ghr^{-/-};Mdr2^{-/-} mice develop a severe liver fibrosis phenotype

Analysis of hematoxylin and eosin stainings (HE) revealed disruption of liver architecture in Ghr^{-/-}; Mdr2^{-/-} mice compared to all control animals. Additionally, CAB staining analysis of liver sections showed a higher degree of collagen deposition resembling bridging fibrosis in Ghr^{-/-};Mdr2^{-/-} mice compared to all controls (Fig. 2A and Supporting Fig. 1A). Surprisingly, we did not observe increases of *Col1a1* and *Col3a1* mRNA expression in Ghr^{-/-};Mdr2^{-/-} mice compared to Mdr2^{-/-} animals (Supporting Fig. 1B). Nevertheless, to assess the degree of fibrosis, we analysed whole liver collagen content by measuring hydroxyproline levels which revealed Ghr^{-/-};Mdr2^{-/-} mice had a massive increase in hydroxyproline levels compared to all the controls (Fig. 2B). Furthermore, hepatocytes of Ghr^{-/-};Mdr2^{-/-} mice contained less glycogen (as measured by PAS staining), indicating a reduction of hepatocyte function (Fig. 2A). Liver sections stained for the cholangiocyte marker CK19 also showed a higher degree of positive staining in Ghr^{-/-};Mdr2^{-/-} mice compared to all the controls suggesting a greater degree of cholangiocyte proliferation (Fig. 2A). Overall, these findings indicate that Ghr^{-/-};Mdr2^{-/-} mice have strongly aggravated fibrotic phenotype typified by extensive collagen deposition, cholangiocyte proliferation and bile acid homeostasis deregulation.

Ghr^{-/-};Mdr2^{-/-} mice exhibit up-regulation of pro-fibrogenic factors

To investigate the Ghr^{-/-};Mdr2^{-/-} fibrotic phenotype further, we performed immunohistochemistry to analyse alpha smooth muscle actin (α-SMA) levels, a marker of activated hepatic stellate cells (HSC). Livers of Ghr^{-/-};Mdr2^{-/-} mice demonstrated increased α-SMA staining (Fig. 3A). This result was further confirmed by qRT-PCR and immunoblot analysis of whole liver homogenates, both demonstrating marked increases in *αSma* mRNA expression and α-SMA protein levels in Ghr^{-/-};Mdr2^{-/-} mice compared to controls (Fig. 3B and 3C). Interestingly, α-SMA protein levels were also increased in Ghr^{-/-} mice compared to Wt mice (as measured by immunohistochemistry, western blot and qRT-PCR (Fig. 3A, 3B and 3C), and the average of the latter two methods (Supporting Fig. 1C). This could indicate a larger reservoir of activated HSC residing in livers lacking Ghr^{-/-}. Additionally, transcription of characteristic pro-fibrogenic factors, such as *Pdgfrβ*, *Pdgfrβ*, *Tgfβ*, *Tgfβr1* and *Tnfa* were highly up-regulated in Ghr^{-/-};Mdr2^{-/-} mice (Fig. 3D). In addition, livers of Ghr^{-/-};Mdr2^{-/-} mice showed increased levels of p-SMAD2/3 and an enrichment of a gene signature set characteristic of the TGFβ pathway, thus indicating activation of the TGFβ signalling pathway in this genotype (Supporting Fig. 2B and 7A). Furthermore, expression of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), namely *Mmp2*, *Mmp3*, *Mmp14*, *Timp1* and *Timp2*, were up-regulated in Ghr^{-/-};Mdr2^{-/-} mice compared to all the experimental groups (Fig. 3E). Collectively, these data indicate that deletion of *Ghr* in Mdr2^{-/-} mice severely magnifies liver fibrosis in these mice.

Deletion of Ghr sensitizes the hepatocytes to bile acid-induced toxicity

To gain mechanistic insights into the development of liver fibrosis we explored potential differences in proliferation and apoptosis in the livers of experimental mice. Liver sections stained and quantified for Ki67 revealed no significant differences in hepatocyte

proliferation between the experimental groups (Fig. 4A and 4B). However, quantification of Apoptag stainings showed a highly significant increase in hepatocyte apoptosis in *Ghr*^{-/-};*Mdr2*^{-/-} mice relative to all experimental groups (Fig. 4C and 4D). Furthermore, gene set enrichment analysis (GSEA) revealed that livers from *Ghr*^{-/-};*Mdr2*^{-/-} compared to *Mdr2*^{-/-} mice show a significant enrichment of genes associated with apoptosis (Supporting Fig. 7B). This finding prompted us to analyse the expression of genes suggested to play a hepatoprotective (pro-survival) role in hepatocytes such as *Egfr* and *Hnf-6.8–10*. Both genes were shown to be diminished in *Ghr*^{-/-};*Mdr2*^{-/-} and *Ghr*^{-/-} livers relative to control groups (Fig. 4F). Moreover, total EGFR protein was only detectable in Wt and *Mdr2*^{-/-} livers with p-EGFR solely observed in *Mdr2*^{-/-} mice (Fig. 4E and Supporting Fig. 3B). In line, p-ERK, a downstream kinase in the EGFR/GHR pathways,¹¹ was reduced in *Ghr*^{-/-} and *Ghr*^{-/-};*Mdr2*^{-/-} compared to *Mdr2*^{-/-} hepatocytes (Fig. 4E and Supporting Fig. 3A). Additionally, p-AKT remained unchanged in hepatocytes among each genotype (Supporting Fig. 3C), but we observed a reduction in S6K phosphorylation in livers of *Ghr*^{-/-} and *Ghr*^{-/-};*Mdr2*^{-/-} mice compared to control mice (Supporting Fig. 2A). It has been previously shown that toxic bile acids may induce hepatocyte apoptosis, liver injury and contribute the development of liver fibrosis.¹² As mentioned previously, *Ghr*^{-/-};*Mdr2*^{-/-} mice display elevated bile acids levels compared to controls (Fig. 1D). Consequently, we challenged Wt and *Ghr*^{-/-} mice with 1% cholic acid diet to observe if lack of *Ghr* causes increased liver damage under cholestatic dietary conditions. Serum parameters (ALP, AST and ALT) were significantly elevated in *Ghr*^{-/-} mice compared to Wt littermates fed with cholic acid (Fig. 5A). Histological analysis of HE liver sections revealed necrotic areas and bile duct infarcts in *Ghr*^{-/-} mice, whereas Wt controls displayed a largely normal liver architecture (Fig. 5B). Furthermore, *Ghr*^{-/-} mice showed increased hepatocyte proliferation and apoptosis demonstrated by Ki67 and Apoptag staining and quantification (Fig. 5B and 5C). Additionally, *in vitro* experiments using isolated primary hepatocytes from *Ghr*^{-/-} mice treated with Deoxycholic acid, (DCA), TGFβ or TNFα either alone or in combination, showed a significant reduction in cell viability compared to Wt control cells (Fig. 5D, 5E and 5F). Taken together, these results suggest that *Ghr*^{-/-} animals are more susceptible to both bile acid and cytokine induced liver injury due to a reduction in pro-survival factors.

GHR protects from bile acid-induced ROS in hepatocytes

It has been previously demonstrated that bile acids can induce reactive oxygen species (ROS), oxidative damage, mitochondrial dysfunction and apoptosis in hepatocytes.^{13–15} Therefore, we investigated ROS accumulation in our experimental mice by staining liver sections with an antibody against 4-Hydroxynonenal (4-HNE) (a side product of lipid peroxidation caused by ROS¹⁶). We observed highly increased 4-HNE levels in hepatocytes of *Ghr*^{-/-};*Mdr2*^{-/-} mice compared to all other experimental mice (Fig. 6A and 6B). Furthermore, qRT-PCR analysis of mRNA from whole liver revealed that compared to all other controls, *Ghr*^{-/-};*Mdr2*^{-/-} mice had an up-regulation of genes which are shown to be increased by ROS^{17–19}, including *Nrf2*, *Nqo1*, *Trp53* and its target genes *Noxa* and *Mdm2* (Supporting Fig. 4A), suggesting increased ROS production within the livers of these mice. We confirmed these results by performing immunohistochemical stainings against heme oxygenase 1 (HO-1, *Hmox1*), a marker of increased cellular stress and ROS levels¹⁷. HO-1 was strongly up-regulated in hepatocytes of *Ghr*^{-/-};*Mdr2*^{-/-} mice (Fig. 6C). Furthermore,

qRT-PCR analysis of primary hepatocytes vs. non hepatocytes taken from $Ghr^{-/-};Mdr2^{-/-}$ mice revealed a significantly higher expression of *Nrf2* and its target genes *Hmox1* as well as *Nqo1* (all major regulators activated in response to oxidative stress 20) in the hepatocyte fraction of $Ghr^{-/-};Mdr2^{-/-}$ mice (Fig. 6D). Importantly, 4-HNE staining of liver sections from Wt and $Ghr^{-/-}$ mice fed with a 1% cholic acid enriched diet showed an increase in 4-HNE production in $Ghr^{-/-}$ compared to Wt hepatocytes (Fig. 6E and 6F). These findings indicate that hepatocytes which are lacking *Ghr* are more susceptible to bile acid-induced ROS and ROS induced cell damage.

GHR deletion abrogates liver tumor formation

Our results indicate that $Ghr^{-/-};Mdr2^{-/-}$ have a more severe and advanced liver fibrosis phenotype than $Mdr2^{-/-}$ mice. As fibrosis and cirrhosis can often progress to liver cancer, we further analysed tumor formation in each experimental genotype at 12 months of age. Surprisingly, $Ghr^{-/-};Mdr2^{-/-}$ mice showed a significant decrease in tumor incidence compared to $Mdr2^{-/-}$ mice despite their severe degree of fibrosis (Fig. 7A and 7B). Only 2 out of 10 $Ghr^{-/-};Mdr2^{-/-}$ mice developed tumors, whereas 11 out of 12 $Mdr2^{-/-}$ mice developed tumors. Additionally, tumors from $Mdr2^{-/-}$ mice were larger and more abundant (total tumor number: $Ghr^{-/-};Mdr2^{-/-}$ n = 4; $Mdr2^{-/-}$ n = 50) (Fig. 7A and 7B). Histopathological analysis revealed that $Mdr2^{-/-}$ tumors presented a more solid growth pattern, increased cellular polymorphism but had similar populations of oval cells compared to $Ghr^{-/-};Mdr2^{-/-}$ tumors (Fig. 7F and Supporting Fig. 5A and 5B). This indicates that $Ghr^{-/-};Mdr2^{-/-}$ tumors are less abundant and advanced than $Mdr2^{-/-}$ tumors. Hepatocyte proliferation was increased in non-tumor areas of $Mdr2^{-/-}$ compared to $Ghr^{-/-};Mdr2^{-/-}$ mice, but we did not find differences between both genotypes in the tumor areas (Fig. 7C). Importantly, Apoptag staining revealed a significant increase in apoptosis in both non-tumorigenic tissue and tumorigenic tissue in $Ghr^{-/-};Mdr2^{-/-}$ compared to $Mdr2^{-/-}$ mice (Fig. 7D). Additionally, p-ERK levels were substantially decreased in hepatocytes from non-tumorigenic tissue in $Ghr^{-/-};Mdr2^{-/-}$ compared to $Mdr2^{-/-}$ mice (Supporting Fig. 6A) and slightly decreased within the $Ghr^{-/-};Mdr2^{-/-}$ tumors (Supporting Fig. 6A). EGFR protein expression was absent in tumorigenic and non-tumorigenic hepatocytes in $Ghr^{-/-};Mdr2^{-/-}$ mice (Supporting Fig. 6B), whereas p-AKT levels remained unchanged (Supporting Fig. 6C). Moreover, cell cycle inhibitors and tumor suppressors such as *Cdkn1a*, *Cdkn1b* and *Trp53* were highly up-regulated in $Ghr^{-/-};Mdr2^{-/-}$ mice compared to $Mdr2^{-/-}$ mice (Fig. 7E and Supporting Fig. 4A). In this line, GSEA revealed that $Ghr^{-/-};Mdr2^{-/-}$ livers display an upregulation of genes normally silenced in various kinds of HCCs (Supporting Fig. 7C). These results suggest that impairment of GH signaling exacerbates the liver fibrosis phenotype but decreases tumor development.

Discussion

Clinical symptoms of liver cirrhosis include disturbed energy balance, dysfunction of secondary metabolism and signs of malnutrition.²¹ Dysregulation of several hormones, which control the secondary metabolism, have been associated with liver cirrhosis as is the case in GH resistance syndrome.²¹ This condition manifests itself with high levels of circulating GH and low levels of insulin-like growth factor 1 (IGF-1) and is commonly found in patients suffering from liver cirrhosis.⁵ It has been shown that mice with low or

absent IGF-1 levels develop severe liver fibrosis upon challenge with diverse models of cholestasis.^{22–24} Furthermore, supplementation of recombinant IGF-1 was shown to be therapeutically beneficial in fibrotic animal models²⁵ and pilot clinical trials.^{26,27} Along this line, GH replacement therapy in an adult man suffering from non-alcoholic steatohepatitis (NASH) and fibrosis associated with GH deficiency ameliorated NASH and liver fibrosis.²⁸ Conversely, patients suffering from hypopituitarism develop non-alcoholic fatty liver disease (NAFLD) and cirrhosis.²⁹ Of note, in humans it has been shown that GH resistance is associated with cholestatic diseases, such as primary biliary cirrhosis.³⁰ Here, we provide clear genetic evidence that *Ghr* deletion aggravates liver fibrosis when challenged with genetic or pharmacological cholestatic models. *Ghr*^{-/-};*Mdr2*^{-/-} mice display severe liver injury, as well as high levels of bilirubin and bile acids (Fig. 1D). Furthermore, *Ghr*^{-/-};*Mdr2*^{-/-} mice appear to have an impaired bile acid homeostasis and despite their high levels of circulating bile acids, *Ghr*^{-/-};*Mdr2*^{-/-} mice failed to repress NTCP and Cyp7a1, a phenomena that may contribute to the pronounced cholestatic phenotype³¹ observed in these mice (Supporting Fig. 1C).

Histological examination of liver sections not only revealed severe disruption of the liver architecture and portal-portal-bridging fibrosis, but also impaired hepatocyte function, as PAS stainings revealed an almost complete lack of glycogen within hepatocytes of *Ghr*^{-/-};*Mdr2*^{-/-} mice. Pro-fibrogenic genes were deregulated in *Ghr*^{-/-};*Mdr2*^{-/-} mice. Surprisingly, we found α -SMA positive hepatic stellate cells not only in livers of *Ghr*^{-/-};*Mdr2*^{-/-} and *Mdr2*^{-/-} mice, but also in *Ghr*^{-/-} mice (Fig. 3A, B and C). This suggests that hepatic stellate cells are more susceptible to activation in *Ghr*^{-/-} mice, a scenario that may in part explain the exaggerated collagen deposition observed in livers of *Ghr*^{-/-};*Mdr2*^{-/-} mice. In this context, over-expression of IGF-1 in HSCs protects from liver fibrosis and has been suggested that low levels of IGF-1 may facilitate the activation of hepatic stellate cells *in vivo*.^{32,33}

Of note, hepatocyte proliferation between the four genotypes was unchanged. However, we observed a significant increase in hepatocyte apoptosis in *Ghr*^{-/-};*Mdr2*^{-/-} mice (Fig. 4C and 4D) that may contribute to the development of liver fibrosis^{34,35} in this animal model. Hepatocyte pro-survival factors, such as EGFR, HNF6, IGF1, p-ERK and p-S6K were down-regulated in livers of *Ghr*^{-/-} and *Ghr*^{-/-};*Mdr2*^{-/-} mice, findings that are in line with recent demonstrations by us and others that GHR-STAT5 regulates the expression of EGFR, HNF6 and IGF1 in the liver^{9,11,22}, underpinning the importance of GHR signaling in hepatocytes as a hepato-protective mechanism. Partial hepatectomy experiments have shown that *Ghr*^{-/-} and hepatocyte-specific EGFR knock-outs are impaired in liver regeneration.^{8,36} Moreover, others confirmed that inhibition of EGFR and IGF1R in primary rat hepatocytes showed enhanced cell death when treated with bile acids.³⁷ Thus, deletion of *Ghr* renders hepatocytes more vulnerable to apoptosis-inducing stimuli.

We observed high levels of bile acids in *Ghr*^{-/-};*Mdr2*^{-/-} mice. Furthermore, it has been proposed that bile acids initiate ROS formation in rat hepatocytes.¹⁵ Analysis of ROS levels in our mouse model indeed revealed an increase in oxidative stress in hepatocytes of *Ghr*^{-/-};*Mdr2*^{-/-} mice. We could confirm up-regulation of genes which are expressed upon ROS production. Furthermore, expression analysis of enzymes involved in ROS defence in

isolated hepatocytes versus non hepatocytes confirmed that hepatocytes of $Ghr^{-/-};Mdr2^{-/-}$ mice exhibit elevated oxidative stress (Fig. 6D). Importantly, $Ghr^{-/-}$ mice fed with a bile acid-enriched diet displayed increased liver injury, higher ROS levels and a higher number of apoptotic hepatocytes compared to Wt controls. Moreover, we could show that primary isolated hepatocytes from $Ghr^{-/-}$ mice are more susceptible to bile acids and/or pro-apoptotic cytokine (TNF α and TGF β) treatment compared to Wt cells (Fig. 5D, 5E and 5F). This suggests that hepatocytes lacking GHR signaling may undergo apoptosis in response to a combination of bile acid-induced oxidative stress and pro-apoptotic cytokines such as TGF β and TNF α . $Ghr^{-/-};Mdr2^{-/-}$ mice show a reduced incidence of tumor development at 12 months of age accompanied with significant decrease of proliferating hepatocytes compared to $Mdr2^{-/-}$ mice in non-tumorigenic tissue but exhibited similar hepatocyte proliferation in tumor tissue (Fig 7B and 7C). Conversely, hepatocytes in non-tumorigenic and tumorigenic tissue showed higher apoptosis in $Ghr^{-/-};Mdr2^{-/-}$ compared to $Mdr2^{-/-}$ livers (Fig. 7C and 7D). Furthermore, we observed decreased EGFR and ERK phosphorylation and increased expression of key tumor suppressor genes (*Trp53*, *Cdkn1a* and *Cdkn1b*) in $Ghr^{-/-};Mdr2^{-/-}$ mice, which could possibly contribute to the reduction in tumorigenesis observed in these mice. Thus, $Ghr^{-/-};Mdr2^{-/-}$ may develop fewer tumors due to a loss of pro-survival signals, increased expression of tumor suppressor genes and increased levels of hepatocyte apoptosis.

In agreement with our data, mice bearing the “little” mutation (*Ghrh^{lit}*, a phenocopy of the $Ghr^{-/-}$ model) show reduced DEN-induced liver tumorigenesis³⁹ and importantly, a clinical study revealed no cases of tumor development in a population suffering from a *Ghr* mutation.⁴⁰

Collectively we could confirm an aggravated liver fibrosis phenotype, but decreased tumor development in $Ghr^{-/-};Mdr2^{-/-}$ relative to $Mdr2^{-/-}$ mice signifying the importance of GH signaling in cholestasis-induced liver fibrosis and tumorigenesis. Our findings suggest that GH resistance plays a causal role in the onset and development of cholestasis-induced liver fibrosis and may provide a novel target for the development of new liver fibrosis treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Ilan Stein for providing $Mdr2^{-/-}$ mice; Drs John J. Kopchick and Nadine Binart for providing the $Ghr^{-/-}$ mice; Michaela Schleder for technical assistance; Kristina Müller for helpful discussions; Dr. Dagmar Stoiber for critical reading of the manuscript.

Financial support

Grants: This work was supported by the Austrian Science Fund (FWF): P 25599-B19 to E.C., F3517-B20 to M.T, P25925-B20 to R.E., SFB-F28 to R.E. and M.M. and DK-plus PhD Program Inflammation and Immunity to R.E. And by the Comprehensive Cancer Center Vienna research grant ‘Regulation of cholestasis-induced HCC formation by signal transducer and activator of transcription 3’ to R.E.

Abbreviations

GH	Growth hormone
GHR	Growth hormone receptor
MDR2	Multi-drug resistance 2
HNF6	Hepatocyte nuclear factor 6
EGFR	Epidermal growth factor receptor ALP - alkaline phosphatase
IGF1	Insulin like growth factor
ROS	Reactive oxygen species
HCC	Hepatocellular carcinoma
TGF-β	Transforming growth factor beta
PDGF	Platelet-derived growth factor
MCP-1	Monocyte chemoattractant protein-1
HSC	Hepatic stellate cells
IL-6	Interleukin 6
TNF-α	Tumor necrosis factor alpha
ECM	Extracellular matrix
JAK2	Janus kinase 2
STAT5	Signal transducer and activator of transcription 5
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ALS	Acid labile subunit
mRNA	Messenger RNA
IGFBP1	Insulin like growth factor binding protein 1
ALP	Alkaline phosphatase
AST	Aspartate amino transferase
ALT	Alanine aminotransferase
HE	Hematoxylin-eosin
CAB	Chromotrope aniline blue
PAS	Periodic acid-Schiff
α-SMA	Alpha smooth muscle actin

MMP	Matrix metalloproteinase
TIMP	Tissue inhibitor of metalloproteinases
GSEA	Gene set enrichment analysis
ERK	Extracellular-signal Regulated Kinase
DCA	Deoxycholic acid
HO-1	Heme oxygenase 1

References

- Hui AY, Friedman SL. Molecular basis of hepatic fibrosis. *Expert Rev Mol Med*. 2003; 5:1–23. [PubMed: 14987408]
- Battaller R, Brenner DA. Liver fibrosis. *J Clin Invest*. 2005; 115:209–218. [PubMed: 15690074]
- Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. *Comp Hepatol*. 2007; 6:7. [PubMed: 17663771]
- Baik M, Yu JH, Hennighausen L. Growth hormone-STAT5 regulation of growth, hepatocellular carcinoma, and liver metabolism. *Ann N Y Acad Sci*. 2011; 1229:29–37. [PubMed: 21793836]
- Bonefeld K, Möller S. Insulin-like growth factor-I and the liver. *Liver Int Off J Int Assoc Study Liver*. 2011; 31:911–919.
- Zhou Y, et al. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci U S A*. 1997; 94:13215–13220. [PubMed: 9371826]
- Fickert P, et al. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. *Gastroenterology*. 2006; 130:465–481. [PubMed: 16472600]
- Natarajan A, Wagner B, Sibia M. The EGF receptor is required for efficient liver regeneration. *Proc Natl Acad Sci U S A*. 2007; 104:17081–17086. [PubMed: 17940036]
- Lahuna O, et al. Involvement of STAT5 (signal transducer and activator of transcription 5) and HNF-4 (hepatocyte nuclear factor 4) in the transcriptional control of the hnf6 gene by growth hormone. *Mol Endocrinol Baltim Md*. 2000; 14:285–294.
- Wang M, et al. Transcriptional activation by growth hormone of HNF-6-regulated hepatic genes, a potential mechanism for improved liver repair during biliary injury in mice. *Am J Physiol - Gastrointest Liver Physiol*. 2008; 295:G357–G366. [PubMed: 18511741]
- González L, et al. GH modulates hepatic epidermal growth factor signaling in the mouse. *J Endocrinol*. 2010; 204:299–309. [PubMed: 20032199]
- Perez M-J, Briz O. Bile-acid-induced cell injury and protection. *World J Gastroenterol WJG*. 2009; 15:1677–1689. [PubMed: 19360911]
- Rodrigues CM, Fan G, Wong PY, Kren BT, Steer CJ. Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production. *Mol Med Camb Mass*. 1998; 4:165–178. [PubMed: 9562975]
- Yerushalmi B, Dahl R, Devereaux MW, Gumprecht E, Sokol RJ. Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. *Hepatol Baltim Md*. 2001; 33:616–626.
- Fang Y, et al. Bile acids induce mitochondrial ROS, which promote activation of receptor tyrosine kinases and signaling pathways in rat hepatocytes. *Hepatol Baltim Md*. 2004; 40:961–971.
- Comporti M. Lipid peroxidation and biogenic aldehydes: from the identification of 4-hydroxynonenal to further achievements in biopathology. *Free Radic Res*. 1998; 28:623–635. [PubMed: 9736314]
- Aleksunes LM, Manautou JE. Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease. *Toxicol Pathol*. 2007; 35:459–473. [PubMed: 17562481]

18. Eno CO, Zhao G, Olberding KE, Li C. The Bcl-2 proteins Noxa and Bcl-xL co-ordinately regulate oxidative stress-induced apoptosis. *Biochem J.* 2012; 444:69–78. [PubMed: 22380599]
19. Montero J, Dutta C, van Bodegom D, Weinstock D, Letai A. p53 regulates a non-apoptotic death induced by ROS. *Cell Death Differ.* 2013; 20:1465–1474. [PubMed: 23703322]
20. Itoh K, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999; 13:76–86. [PubMed: 9887101]
21. Blomsma MC, de Knecht RJ, Dullaart RP, Jansen PL. Insulin-like growth factor-I in liver cirrhosis. *J Hepatol.* 1997; 27:1133–1138. [PubMed: 9453443]
22. Blaas L, et al. Disruption of the growth hormone-Signal transducer and activator of transcription 5-Insulinlike growth factor 1 axis severely aggravates liver fibrosis in a mouse model of cholestasis. *Hepatology.* 2010; 51:1319–1326. [PubMed: 20162728]
23. Mair M, et al. Signal transducer and activator of transcription 3 protects from liver injury and fibrosis in a mouse model of sclerosing cholangitis. *Gastroenterology.* 2010; 138:2499–2508. [PubMed: 20193684]
24. Mair M, Blaas L, Österreicher CH, Casanova E, Eferl R. JAK-STAT signaling in hepatic fibrosis. *Front Biosci Landmark Ed.* 2011; 16:2794–2811. [PubMed: 21622209]
25. Castilla-Cortazar I, et al. Hepatoprotective effects of insulin-like growth factor I in rats with carbon tetrachloride-induced cirrhosis. *Gastroenterology.* 1997; 113:1682–1691. [PubMed: 9352873]
26. Conchillo M, et al. Insulin-like growth factor I (IGF-I) replacement therapy increases albumin concentration in liver cirrhosis: results of a pilot randomized controlled clinical trial. *J Hepatol.* 2005; 43:630–636. [PubMed: 16024131]
27. Conchillo M, Prieto J, Quiroga J. [Insulin-like growth factor I (IGF-I) and liver cirrhosis]. *Rev Esp Enfermedades Dig Organo Of Soc Esp Patol Dig.* 2007; 99:156–164.
28. Takahashi Y, et al. Growth hormone reverses nonalcoholic steatohepatitis in a patient with adult growth hormone deficiency. *Gastroenterology.* 2007; 132:938–943. [PubMed: 17324404]
29. Adams LA, Feldstein A, Lindor KD, Angulo P. Nonalcoholic fatty liver disease among patients with hypothalamic and pituitary dysfunction. *Hepatol Baltim Md.* 2004; 39:909–914.
30. Sookoian S, Pirola CJ. Genetic determinants of acquired cholestasis: a systems biology approach. *Front Biosci Landmark Ed.* 2012; 17:206–220. [PubMed: 22201739]
31. Halilbasic E, Claudel T, Trauner M. Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *J Hepatol.* 2013; 58:155–168. [PubMed: 22885388]
32. Sanz S, et al. Expression of insulin-like growth factor I by activated hepatic stellate cells reduces fibrogenesis and enhances regeneration after liver injury. *Gut.* 2005; 54:134–141. [PubMed: 15591519]
33. Allan GJ, Beattie J, Flint DJ. Epithelial injury induces an innate repair mechanism linked to cellular senescence and fibrosis involving IGF-binding protein-5. *J Endocrinol.* 2008; 199:155–164. [PubMed: 18676497]
34. Guicciardi ME, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. *Gut.* 2005; 54:1024–1033. [PubMed: 15951554]
35. Wang K, Lin B. Pathophysiological Significance of Hepatic Apoptosis. *ISRN Hepatol.* 2013:1–14.
36. Zerrad-Saadi A, et al. GH Receptor Plays a Major Role in Liver Regeneration through the Control of EGFR and ERK1/2 Activation. *Endocrinology.* 2011; 152:2731–2741. [PubMed: 21540290]
37. Dent P, et al. Inhibition of insulin/IGF-1 receptor signaling enhances bile acid toxicity in primary hepatocytes. *Biochem Pharmacol.* 2005; 70:1685–1696. [PubMed: 16207485]
38. Moshage H. The cirrhotic hepatocyte: navigating between Scylla and Charybdis. *J Hepatol.* 2004; 40:1027–1029. [PubMed: 15158346]
39. 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. [PubMed: 11698349]
40. Guevara-Aguirre J, et al. Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. *Sci Transl Med.* 2011; 3:70ra13.

Figure 1.

Ghr deletion combined with loss of *Mdr2* increases liver damage. (A) Macroscopic analysis reveals significant liver size reduction in *Ghr*^{-/-}; *Mdr2*^{-/-} mice compared to controls. (B) Liver to body weight ratio (LW/BW) was determined in all 4 genotypes (n = 5/genotype). (C) Levels of GH and IGF-1 within the serum were measured by ELISA and demonstrated GH resistance in *Ghr*^{-/-} and *Ghr*^{-/-}; *Mdr2*^{-/-} mice (n = 6/genotype). (D) Analysis of serum parameters revealed a significant increase of AST, ALT, ALP, bilirubin and bile acids in

Ghr^{-/-};Mdr2^{-/-} mice relative to all controls (n = 5/genotype). Results are shown as mean ± standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GH, growth hormone; IGF-1, insulin like growth factor 1

Figure 2.

Ghr^{-/-};Mdr2^{-/-} mice develop a severe liver fibrosis phenotype. (A) Histological and immunohistochemical analysis of liver sections from mice at 8 weeks of age stained with HE, CAB, PAS and CK19. Ghr^{-/-};Mdr2^{-/-} mice showed disrupted liver architecture, increased collagen deposition and cholangiocyte proliferation as well as severe glycogen reduction in hepatocytes compared to all controls. (B) Hydroxyproline was measured in livers from 8 weeks old mice (n = 6/genotype). Results are shown as mean ± standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.

HE, hematoxylin-eosin; CAB, chromotrope aniline blue; PAS, periodic acid-Schiff; CK-19, cytokeratin 19.

Figure 3.

$Ghr^{-/-};Mdr2^{-/-}$ mice display up-regulation of profibrogenic growth factors, cytokines and extracellular matrix remodeling factors. (A) Immunohistochemical analysis of liver sections taken from mice at 8 weeks of age stained for α -SMA. (B) Representative western blot analysis of α -SMA levels in whole liver homogenates indicates increased activated hepatic stellate cells in livers from $Ghr^{-/-}$, $Mdr2^{-/-}$ and $Ghr^{-/-};Mdr2^{-/-}$ mice. HSC70 served as a loading control. (C) Relative mRNA levels of α Sma were quantified by qRT-PCR from mouse livers at 8 weeks of age and normalized to *Gapdh* (n = 4/genotype). (D) *Pdgfr β* ,

Pdgfr β , *Tgf β* , *Tgf β 1* and *Tnfa* genes showed significantly increased mRNA levels in *Ghr*^{-/-};*Mdr2*^{-/-} mice compared to all controls. Relative mRNA levels were quantified by qRT-PCR from livers of 8 weeks old mice and normalized to *Gapdh* (n = 4/genotype). (E) mRNA levels of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) were extensively increased in *Ghr*^{-/-};*Mdr2*^{-/-} mice, suggesting a continuous remodelling of the extracellular matrix. Relative mRNA levels were quantified by qRT-PCR from livers of 8 weeks old mice and normalized to *Gapdh* (n = 4/genotype). Results are shown as mean $\pm$ standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001. α -SMA, alpha-smooth muscle actin; HSC70, Heat shock protein 70

Figure 4.

Ghr^{-/-};Mdr2^{-/-} mice show a down-regulation in hepato-protective factors accompanied by an increase in hepatocyte apoptosis. (A) Immunohistochemical analysis of liver sections from mice at 8 weeks of age stained for Ki67. (B) Quantification of Ki67 positive hepatocytes showed no significant difference between Mdr2^{-/-} and Ghr^{-/-};Mdr2^{-/-} mice (n = 5/genotype). (C) Immunohistochemical staining of liver sections stained with Apoptag shows an increase in hepatocyte apoptosis in Ghr^{-/-};Mdr2^{-/-} mice. (D) Quantification of hepatocytes undergoing apoptosis seen in (C) (n = 5/genotype). (E) Representative western blot analysis of whole

liver homogenates from 8 week old mice revealed activation of p-EGFR only in *Mdr2*^{-/-} mice and a down-regulation of EGFR, p-ERK and ERK in *Ghr*^{-/-} and *Ghr*^{-/-};*Mdr2*^{-/-} livers. HSC70 served as a loading control. (F) Relative mRNA levels of *Egfr* and *Hnf6* were quantified by qRT-PCR from mouse livers at 8 weeks of age and normalized to *Gapdh* (n 4/genotype). Results are shown as mean ± standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.

EGFR, epidermal growth factor receptor; p-ERK, phospho-extracellular-signal regulated kinase; ERK, extracellular-signal regulated kinase; HSC70, heat shock protein 70

Figure 5.

Hepatocytes of Ghr^{-/-} mice are more susceptible to bile acid induced damage *in vivo* and *in vitro* compared to Wt littermate controls. (A) Ghr^{-/-} and Wt mice were fed with a 1% cholic acid enriched diet. Serum levels of ALS, AST and ALP were significantly elevated in Ghr^{-/-} mice compared to Wt mice, suggesting increased liver damage in Ghr^{-/-} mice. (B) Immunohistochemical analysis of liver sections from Ghr^{-/-} and Wt mice fed with a 1% cholic acid enriched diet stained with HE, ki67 and Apoptag. (C) Quantification of Ki67 and Apoptag positive hepatocytes from mice fed with a diet containing 1% cholic acid showed

increased apoptosis and proliferation in $Ghr^{-/-}$ mice relative to Wt littermates. ($n = 5$ /genotype). (D) Primary hepatocytes from Wt and $Ghr^{-/-}$ mice were treated with 200 μ m or 250 μ m DCA for 4 hours. $Ghr^{-/-}$ hepatocytes showed a significant decrease in cell viability compared to Wt hepatocytes. (E) Primary hepatocytes were treated with either 20ng/ μ l TNF α alone for 20 hours (left panel) or in combination with 250 μ m DCA for 3 hours (right panel). (F) Primary hepatocytes were treated with either 10ng/ μ l TGF β alone for 48 hours (left panel) or in combination with 250 μ m DCA for 3 hours (right panel) followed by MTT cell viability assay. Results are shown as mean $\pm$ standard error of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HE, hematoxylin-eosin; DCA, deoxycholic acid

Figure 6.

Hepatocytes of Ghr^{-/-};Mdr2^{-/-} mice show an increase of ROS production due to toxic bile acid accumulation. (A) Immunohistochemical analysis of liver sections from mice at 8 weeks of age stained with an antibody against 4-HNE. (B) Quantification of 4-HNE positive hepatocytes from (A) showed severe increase of lipid peroxidation in hepatocytes of Ghr^{-/-};Mdr2^{-/-} mice (n = 5/genotype). (C) Immunohistochemical analysis of liver sections from mice at 8 weeks of age stained with an antibody against HO-1. (D) Relative mRNA levels of *Nrf2*, *Nqo1* and *Hmox1* were quantified by qRT-PCR in isolated hepatocytes

compared to non-hepatocytes from 8 week old mice and normalized to *Gapdh* (n = 5/genotype). (E) Liver sections of *Ghr*^{-/-} and Wt mice fed with a cholestatic diet containing 1% cholic acid stained with an antibody against 4-HNE reveals increased lipid peroxidation in hepatocytes of *Ghr*^{-/-} mice. (F) Quantification of 4-HNE staining from (E). Results are shown as mean $\pm$ standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001. ROS, reactive oxygen species; 4-HNE, 4-hydroxynonenal; HO-1, heme oxygenase 1

Figure 7.

Deletion of *Ghr* suppresses liver tumorigenesis. (A) Macroscopic pictures of livers of experimental mice show numerous tumors in *Mdr2*^{-/-} mice compared to *Ghr*^{-/-};*Mdr2*^{-/-} mice at 12 months of age. (B) Quantification of tumor number/mouse in all four genotypes. Each data point represents one animal (n = 10/genotype). (C) Immunohistochemical analysis of liver sections from mice at 12 months of age stained with ki67 showed increased hepatocyte proliferation in *Mdr2*^{-/-} relative to *Ghr*^{-/-};*Mdr2*^{-/-} mice at the age of 12 months in non-tumor areas, but no differences in cell proliferation rates were observed in tumorigenic tissues from

both genotypes (n = 10/genotype) (T: tumor, NT: non-tumor area). (D) Apoptag staining indicated increased apoptosis in $Ghr^{-/-};Mdr2^{-/-}$ non-tumorigenic and tumorigenic tissue at 12 months compared to $Mdr2^{-/-}$ mice (n = 10/genotype) (T: tumor, NT: non-tumor area). (E) mRNA levels of cell cycle inhibitors *Cdkn1a* and *Cdkn1b* were significantly increased in $Ghr^{-/-};Mdr2^{-/-}$ mice compared to controls in isolated hepatocytes of mice at 8 weeks of age (n = 5/genotype). (F) Tumors from $Ghr^{-/-};Mdr2^{-/-}$ mice are less advanced compared to $Mdr2^{-/-}$ tumors. Tumor grading reveals that $Mdr2^{-/-}$ mice develop more tumors with cellular polymorphism and solid growth pattern. Results are shown as mean $\pm$ standard error of the mean. *P < 0.05; **P < 0.01; ***P < 0.001.