Hepatocellular alterations and dysregulation of oncogenic pathways in the liver of transgenic mice overexpressing growth hormone

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Summary german

Wachstumshormon- (GH) Überexpression in transgenen Mäusen ist in hohem Alter mit der Entwicklung von Lebertumoren assoziert. Die präneoplastische Pathologie, die in der Leber von jungen erwachsenen GH-überexprimierenden Mäusen zu beobachten ist, ähnelt der in Patienten mit hohem Risiko für Leberkrebs. Zur Aufklärung der zugrunde liegenden proonkogenen Prozesse der Leberpathologie, induziert durch lang andauernde Einwirkung von erhöhten Wachstumshormonspiegeln, wurde die Aktivierung und Expression mehrerer Komponenten verschiedener Signalwege in jungen erwachsenen GH-überexprimierenden Mäusen ausgewertet, die an der Entstehung von Leberkrebs beteiligt sind. Zusätzlich wurden Männchen und Weibchen parallel analysiert, um möglichen Geschlechtsdimorphismus zu beurteilen. Transgene Mäuse beider Geschlechter wiesen eine Hypertrophie der Hepatozyten auf mit vergrößerten Zellkernen und erhöhter Proliferation, die sich verstärkt bei den Männchen zeigte. Zum weiteren Verständnis der Entwicklung von prä- zu neoplastischen Läsionen, erfolgten Untersuchungen bezüglich der Unterschiede molekularer hepatozellulärer Veränderungen zwischen tumorösem und nicht-tumorösem Gewebe in alten GH-überexprimierenden Mäusen. Es wurde eine lang anhaltende Dysregulation mehrerer onkogener Signalwege in der Leber von GH-überexprimierenden transgenen Mäusen beobachtet, die sich von jungen erwachsenen bis hin zu alten Tieren erstreckt. Mehrere Signalmediatoren und Effektoren in den transgenen Mäusen waren hochreguliert im Vergleich zu ihren normalen Kontrollen, einschließlich Akt2, NF-κB, GSK3-β, β-catenin, cyclin D1, cyclin E, c-myc, c-jun und c-fos. Die beschriebenen molekularen Veränderungen zeigten keinen Geschlechtsdimorphismus innerhalb der transgenen Mäuse, bis auf nukleäre Lokalisation von Cyclin D1 bei Männchen.

Wir schließen daraus, dass lang anhaltende Exposition von GH Veränderungen in Signalwegen der Leberzellen induziert, die beteiligt sind an Zellwachstum, Proliferation und Überleben der Zellen, die jenen Veränderungen ähneln, die in vielen menschlichen Lebertumoren gefunden wurden.

Index

Abbreviations & Symbols

AP-1	Activating-Protein-1
APC	Adenomatous-Polyposis-Coli Protein
BCA	Bicinchoninic acid Assay
BSA	Bovine Serum Albumine
Catenin	Cadherin-Associated Protein
CDK	Cyclin-Dependant-Kinases
c-Fos	cellular-FBJ murine osteosarcoma
c-Jun	cellular-protein product of v-jun avian sarcoma virus 17 oncogene homolog
CK1	Casein Kinase I
c-Myc	cellular-Myelocytomatose
CRE	cAMP-Response Element
Ctrl	Control
DAB	Diaminobenzidine
DNA	Desoxyribonucleic Acid
DNA-PK	DNA-dependent Protein Kinase
E2F	E2 Transcription Factor
EASL	European Association for the Study of the Liver
E-cadherin	Epithelial Calcium Dependant Adherin
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
Epo	Erythropoietin
ERK	Extracellular-signal Regulated Kinase
EtOH	Ethanol
Fz receptor	Frizzled receptor
g	Gram
G1	Gap1
G2	Gap2
G-CSF	Granulocyte-Colony Stimulating Factor
GH	Growth Hormone
GHR	Growth Hormone Receptor
GHRH	Growth-Hormone-Releasing-Hormone
GP	Glycoprotein
GSK3	Glykogensynthase-Kinase
H&E	Hematoxylin and Eosin
H_2Od	Distilled Water
HCC	Hepatocellular Carcinoma
HCl	Hydrochloric Acid
HEPES	4-2-Hydroxyethyl-1-Piperazineethanesulfonic Acid
HGF	Hepatocyte Growth Factor
hGH	Human Growth Hormone
HRP	Horseradish Peroxidase
IGF1	Insulin-like Growth Factor 1
IgG	Immunoglobulin

IHQ	Immunohistochemistry
IKK	IκBα-Kinase-Komplex
IL	Interleukin
IRS 1/2	Insulin Receptor Substrate 1/2
IκB	Inhibitor of κ Binding
JAK2	Janus Kinase 2
kDa	kilo Dalton
LPS	Lipopolysaccharide
M	Molar
mA	Milliampere
MAPkinase	Mitogen-Activated Protein Kinase
MEK	Mitogen-activated Protein Kinase Kinase
mg	Milligram
ml	Milliliter
mM	Millimolar (millimol/liter)
M-phase	Mitosis-phase
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target Of Rapamycin
mTORC1	Mammalian Target Of Rapamycin Comlex 1
mTORC2	Mammalian Target Of Rapamycin Comlex 2
N	Normal
NaCl	Sodium Chloride
NAFLD	Non-Alcoholic Fatty Liver Disease
Neg	Negative
NF	Normal Female
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
NLS	Nuclear Localization Signal
NM	Normal Male
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PDPK1	Phosphoinositide Dependent Protein Kinase-
PEPCK-bGH	Phosphoenolpyruvate Carboxykinase-bovine Growth Hormone
PH	Pleckstrin Homology
pH	measure of the acidity or basicity of a solution, it is defined as the decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
PI3K	Phosphatidylinositol 3 Kinase
PIAS	Protein Inhibitor of Activated STAT
PIP	PCNA-Interacting Protein
PIP2	Phosphatidylinositol (4,5)-bis-Phosphate
PIP3	Phosphatidylinositol (3,4,5)-tris-Phosphate
Akt/PKB	Protein Kinase B
PMSF	Phenylmethylsulfonyl Fluoride
PP2A	Protein Phosphatase 2A
pRB	Retinoblastom Protein
Prl	Prolactin

PTEN	Phosphatase and Tensin Homolog
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene Fluoride
Raf	Mitogen-activated Protein Kinase Kinase Kinase
Ras	Rat Sarcoma
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of Measurement
Ser	Serine
SFK	Src Family Kinase
SH2	Src-Homology 2
SOCS	Suppressor Of Cytokine Signalling
S-phase	Synthesis-phase
Src	Sarcoma
STAT	Signal Transducer and Activator of Transcription
STH	Somatotropin
TACE	Transarterial Chemoembolization
TBS	Tris-buffered Saline
TCF/LEF	T-cell specific Transcription Factor/Lymphoid Enhancer-binding Factor
TCF	T-cell Factor
TF	Transgenic Female
Tg	Transgenic
Thr	Threonine
TM	Transgenic Male
TNFα	Tumor Necrose Factor α
Tpo	Thrombopoietin
TRE	TPA-responsive Promoter Elements
TRE	Transcriptional Regulatory Element
TSC 1/2	Tuberous Sclerosis Complex 1/2
Tu	Tumour
v/v	soluted volume (ml) in 100 ml of solution
w/v	soluted mass (g) in 100 ml of solution
WB	Western Blotting
Wnt	Wingless – Int 1
Wt	Weight
xg	acceleration due to gravity at the surface of the earth (9,806 m/s^2)
Y	Tyrosine
β-TrCP	beta-Transducin repeat-Containing Protein
µg	Microgram
µm	Micrometer
%	Percent
°C	Degree Celsius
2-ME	2-Mercaptoethanol
2x	Twofold

1. Introduction

1.1. Growth Hormone

1.1.1. Growth Hormone regulation

Growth hormone (GH), also known as Somatotropin (STH), is synthesized by somatotrophic α cells in the anterior pituitary gland. The pulsatile release of the 191 amino acid polypeptide is regulated via Growth-Hormone-Releasing-Hormone (GHRH / Somatoliberin) and Somatostatin (Somatotropin-Inhibitory Hormone / SIH) through the hypothalamus. Apart from the hypothalamus, Somatostatin is released from the pancreas during digestion. Beyond this, GH secretion is stimulated by ghrelin, an ingestion regulating protein from the parietal cells of the stomach lining. The pulsatile release of GH from the pituitary gland is different in males and females. In male rats GH pulses emerge in 3- to 4-hour intervalls with small level inbetween the peaks. Female rats in contrast exhibit a more continuous secretion (Jansson et al., 1985). Analysis in humans revealed a related pattern with men having large nocturnal GH pulses and small pulses during the day, while women show more frequent, consistent pulses and in general a more continuous GH secretion (Jaffe et al., 1998).

1.1.2. Physiological Actions of Growth Hormone

GH is a main modulator of body growth and metabolism. At the cellular level it regulates cell proliferation, differentiation, survival and motility. The main anabolic effects of GH imply increase of height, mineralization of bones, increase of muscle mass and protein synthesis, stimulation of the immune system and of growth of internal organs, elevation of lipolysis, reduction of glucose uptake and promotion of gluconeogenesis in the liver. GH acts directly on target cells or indirectly via induction of insulin-like growth factor 1 (IGF1 – somatomedin C) synthesis in the liver and a big variety of extra-hepatic tissue. The liver is the main source for circulating IGF-1, but in muscle for example the local production of IGF-1 is of big importance in autocrine and paracrine signaling. On the hypothalamic – pituitary axis IGF-1 acts as negative feedback for GH secretion (Vijayakumar et al., 2010; Lichanska and Waters, 2008).
Many of the effects controlled through GH are opponent to the effects controlled through insulin.

Lipolysis in the adipose tissue for example is induced by an increase of the hormone-sensitive lipase (HSL) activity. Therefore suppression of insulin action on adipocytes might be controlled via inhibition of glucose uptake or upregulation of the expression of a regulatory subunit of the phosphoinositol-3-kinase (PI3K) of the PI3K/AKT pathway. The protection of proteins via inhibtion of proteolysis and stimulation of protein synthesis might be an IGF-1-dependent effect of GH (Vijayakumar et al., 2010). The interplay between GH, IGF-1 and Insulin is very complex and subject of current research.

1.1.3. Growth Hormone Receptor and signal transduction

The GH receptor (GHR) is part of the class 1 cytokine receptor family, including erythropoietin, prolactin, granulocyte colony stimulating factor, leptin and various interleukins (Waters et al., 1999). Its polypeptide chain crosses the plasma membrane only once, and consists of an extracellular domain for ligand binding, a transmembrane domain and an intracellular domain for signal transduction. In the cytoplasmic domain tyrosine residues are accessible for phosphorylation, which plays a central role in signal transduction (Bazan, 1990; Waxman and Frank, 2000) (Figure 1).

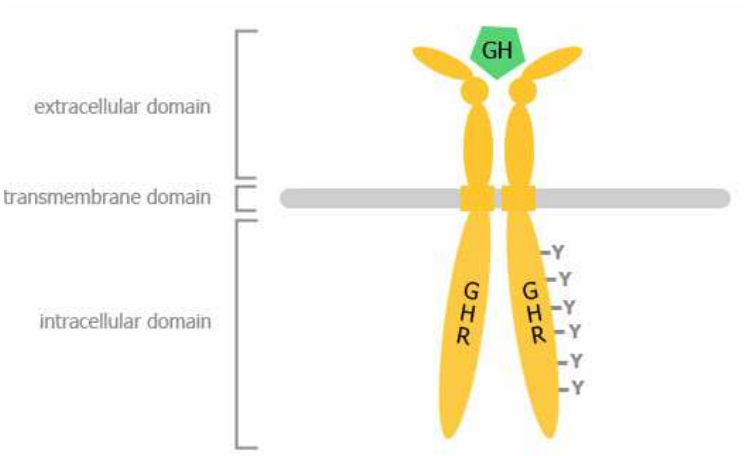

Figure 1. Structure of GHR. Individual domains of the GHR (Growth Hormone Receptor) are shown. The intracellular domain contains the tyrosine (Y) residues for phosphorylation and thereby activation of the receptor. Scheme is adapted from Frank, 2002.

In contrary to former assumption the GHR is predominantly constitutively dimerized without GH stimulus. Hormone binding induces a conformational change of the

dimeric structure in form of subunit rotation leading to alignment of the tyrosine-protein kinases Janus kinases 2 (JAK2) and Src family kinases (SFKs) linked to the proline rich box 1 motif of the intracellular domain of the receptor. Via mutual autophosphorylation of the JAKs and further phosphorylation of the intracellular domains of the GHR, receptor activation is concealed. Signal mediation to the nucleus is generated via signal transducers and activators of transcription (STATs) and the phosphatidylinositol 3 kinase (PI3K)/Akt pathway (Brooks and Waters, 2010; Xu and Messina, 2009; Lichanska and Waters, 2008; Waters et al., 1999). SFK activation is independent from JAK2 activation and leads to signal mediation via the MAPK/ERK pathway (Brooks and Waters, 2010; Darnell et al., 1994). In addition GH is involved in the modulation of the expression and activity of the epidermal growth factor receptor (EGFR), which induces these pathways as well (Jansson et al., 1998; Ekberg et al., 1989; Miquet et al., 2008).

1.1.4. Diseases due to failure of GH regulation

A long term study with patients carrying a mutation in the GHR gene leading to severe GH and IGF-1 deficiencies, the so called Laron syndrome, indicated an explicit decrease of cancer and type 2 diabetes in comparison to normal controls; however, life expectancy was not extended in this cohort, unlike animal models with mutations in the IGF-1 pathway (Guevara-Aquirre et al., 2011). Patients with Laron syndrome are characterized by dwarfism, truncal obesity, delayed puberty, recurrent hypoglycemia and facial abnormalities. Failure of GH production in the pituitary gland mostly due to pituitary and parasellar tumors leads to growth failure in children and to poor bone density and dysfunction of the lipid and protein metabolism in adults, to name the major symptoms.

On the contrary, human growth hormone excess, before closure of the growth plate of bones, results in gigantism (hypersomie) in children. Children grow in height, accompanied by growth of muscles and organs. These patients suffer from headache, increased sweating, sleep problems, delayed puberty, irregular menstruation, weakness and thickening of facial features amongst others. In adults GH excess leads to acromegaly, characterized by large feet, large hands, large jaw (prognathism), large tongue (macroglossia), sleep apnea, thickening of the skin, carpal tunnel syndrome,

easy fatigue and decreased muscle strength. The body height does not increase if GH excess occurs in adulthood. Mostly this disease pattern is due to a GH-secreting pituitary adenoma, rarely as a consequence of ectopic secretion of growth-hormone-releasing hormone (GHRH) with pituitary hyperplasia (Herder, 2012; Chanson and Salenave, 2008). Ultimately pegvisomant, a growth hormone receptor antagonist approved for therapy in patients with acromegaly, moves into focus as an anti-cancer therapeutic, as the involvement of the GH/IGF-1 axis has been implied in the progression of several cancer entities (Kopchick et al., 2014).

1.1.5. GH as a drug

Therapeutic use of human GH (hGH) was initiated in growth hormone deficiency from 1958 on, using hGH from the pituitary of human cadaver. Until the possibility of producing recombinant hGH in 1985 its use was limited. The list of indications for hGH therapy has risen so far. Apart from GH-deficiency states in children and adults, there is a range of non-GH-deficiency states approved for hGH therapy, like children born small for gestational age (Simon et al., 2008) or Idiopathic short stature (ISS) (Bryant et al., 2007). Further indications display Turner syndrome (Davenport et al., 2007), SHOX (short stature homeobox gene) deficiency (Blum et al., 2007), Prader-Willi syndrome (Goldstone et al., 2008), Noonan syndrome (Romano et al., 2009) and AIDS wasting (Kemp and Frindik, 2011). Beyond that hGH replacement therapy in adult GH deficiency improves cardiovascular risk and function (Widdowson and Gibney, 2008; Burger et al., 2006). Most side effects are local reactions due to subcutaneous injection and mostly self-limiting like headache, nausea and fever. Frequent adverse events of hGH therapy observed in adults are edema and carpal tunnel syndrome and particularly in children are transient idiopathic intracranial hypertension (IIH, known as pseudotumor cerebri), gynecomastia and slipped capital femoral epiphysis (Kemp and Frindik, 2011). As acromegaly is known to increase the risk of developing colorectal cancer (Renehan and Brennan, 2008), it is supposed that hGH therapy might be associated with different malignancies like prostate cancer, breast cancer, Hodgkin´s disease and colorectal cancer. That illustrates the importance of long-term observation of patients with hGH replacement.

1.1.6. GH-transgenic mouse model

In this work a GH overexpressing transgenic phosphoenolpyruvate carboxykinase-bovine growth hormone (PEPCK-bGH) mouse model was used. The fusion gene, microinjected into the male pronucleus, consists of the transcriptional regulatory element (TRE), often referred to as promoter, of the rat phosphoenolpyruvate carboxykinase (PEPCK), connected to the bovine GH (bGH) gene. PEPCK is the velocity determining enzyme of the gluconeogenesis. bGH linked to the PEPCK promoter leads to the expression of the transgene mainly in liver, kidney and adipose tissue. In a more slight degree bGH is expressed in the jejunum of the small intestine, the lung and the mammary gland (McGrane et al., 1990; Kopchick et al., 1999). In the liver bGH is zonally-specific expressed in the periportal compartment mirroring the endogenous PEPCK expression in the liver (McGrane et al., 1990).

Transgenic mice overexpressing GH exhibit increased adult body size, organomegaly and elevated circulating IGF-1 level in combination with other endocrine and metabolic alterations (McGrane et al., 1990; Kopchick et al., 1999; Bartke, 2003; Wanke et al., 1991). The developed hepatomegaly features increased size of hepatocytes and their nuclei as a consequence of hypertrophy and hyperplasia. Old GH-transgenic mice often develop liver tumors (Orian et al., 1989; Orian et al., 1990; Quaife et al., 1989; Snibson et al., 1999; Hoeflich et al., 2001). The connection between GH/IGF-1 levels and the risk of developing cancer is enforced. Elevated circulating GH levels were reported to have mitogenic and anti-apoptotic effects via increase of cell proliferation and survival independent of IGF-1 (Jenkins et al., 2006; Perry et al., 2006). IGF-1 overexpressing mice do not manifest the liver pathology that GH-transgenic mice do. Hence the cytological alterations on hepatocytes in GH-transgenic mice appear as a consequence of the direct effect of GH on the liver, rather than GH action via IGF-1 (Bartke, 2003; Quaife et al., 1989). The preneoplastic pathology in the liver of GH overexpressing mice is similar to that present in humans at high risk of developing hepatic cancer (Snibson, 2002).

1.1.7. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is one of the malignancies most established in the context of chronic inflammation triggering tumor development. The majority of HCC

patients exhibit chronic inflammatory liver disease. Due to the dysregulation of growth of hepatocytes, chronic liver injury results in development of regenerative nodules, dysplastic nodules and HCC (Leonardi et al., 2012; Bruix et al., 2004). As the sixth most common neoplasm worldwide, it reflects 70% - 85% within primary liver cancer cases. Its poor prognosis brings it to the third leading cause of cancer-related mortality worldwide with approximately 600 000 deaths per year. The incidence varies globally with high rates in East Asia and sub-Saharan Africa and lower rates in North America and Europe. This distribution might be a consequence of the prevalence of aetiological risk factors like primarily infection with hepatitis B or hepatitis C virus, mirroring the most common risk factors of HCC (Venook et al., 2010). In the context of the prevalence of aetiological risk factors Aflatoxin B1 should not be excluded. The collected epidemiological data indicates an increase of this disease within the next years. Apart from alcohol abuse, which represents a common trigger for hepatic cirrhosis, upcoming risk factors imply obesity and diabetes leading to non-alcoholic fatty liver disease (NAFLD); (Mittal and El-Seraq, 2013). The mentioned risk factors cause DNA damage in liver cells directly or indirectly via oxidative stress which comes along with liver inflammation. Followed by affected repair mechanisms this impact leads to intensive regeneration and fibrogenesis (Farazi and DePinho, 2006). Several signaling pathways are verifiably activated in hepatocellular carcinogenesis, amongst others PI3K/Akt/mTOR, Wnt/β-catenin, HGF/c-met, EGFR, Ras/Erk. Other cascades like JAK/STAT and Hedgehog are still under study (Llovet and Bruix, 2008; Spangenberg et al., 2009; Zender et al., 2010).

The only possibility for curative treatment for patients with HCC implies early detection of this disease. Optional treatment in early stage HCC involves surgical resection, liver transplantation and radiofrequency ablation. The treatment of advanced stages inevitably is based on palliative therapy including transarterial chemoembolization (TACE) and the multikinase inhibitor Sorafenib, representing the only systemic therapeutical option (EASL, 2012). The lack of success in treatment of advanced stages in HCC and the limited therapeutical options indicate the importance of progress in the research of hepatocarcinogenesis and its curing possibilities. Hence, the general objective of this study was to elucidate the molecular pathogenesis and

signal transduction pathways underlying the pro-oncogenic liver pathology induced by prolonged exposure to elevated GH level.
The following part provides a closer look on the investigated proteins in this work.

1.2. Downstream signaling of GH & investigated proteins

1.2.1. PCNA

PCNA (Proliferating cell nuclear antigen) is a 28.7 kDa protein establishing connections to a lot of proteins involved in the process of replicating DNA, making it valuable as a DNA synthesis marker (Tsurimoto 1999). With its PCNA-interacting proteins (PIPs) it is engaged in DNA replication, DNA repair, cell cycle control, epigenetic inheritance and chromatin metabolism (Maga and Hübscher, 2003). Many of the PIPs contain the so called PIP-box, a common binding motif for PCNA (Jónsson et al., 1998).

1.2.2. Signal Transducers and Activators of Transcription (STATs)

Signal Transducer and Activator of Transcription (STAT) proteins depict the link between extracellular signals like cytokines (interferons, interleukins) and hormones (growth hormone, erythropoetin, prolactin, leptin) and transcription of their target genes. This makes the family of STATs inalienable for immune response, development, differentiation and homeostasis (Darnell et al., 1994). To date, the family of STATs consists of seven members (STAT1, 2, 3, 4, 5a, 5b, 6). In response to the signal they form homo- and heterodimers to reach efficient constellation for further reactions.
Inactive, dephosphorylated STATs are located in the cytoplasm. After ligand binding to the receptor the tyrosine kinases from the Janus family (JAKs) augment their activity via mutual phosphorylation at tyrosine residues, followed by tyrosine phosphorylation of the receptor. This last step composes the binding site for the SH2 (Src-homology 2) domain of the specific STAT (Darnell et al., 1994). The binding of STATs to membrane receptors as well as the dimerization is accomplished via phosphorylated tyrosine residues (Shuai et al., 1994; Schindler and Plumlee, 2008). The phosphorylated motif is detected and linked by the correlated SH2 domain of the partner STAT (Mao et al., 2005). After dimerization and localization to the nucleus via the nuclear localization signal (NLS), STAT dimers bind to STAT response elements, inducing the

transcription of specific genes (Becker et al., 1998; Chen et al., 1998; McBride and Reich, 2003). The Linker Domain forms the essential part between DNA binding and activation of transcription (Yang et al., 1999) (Figure 2).

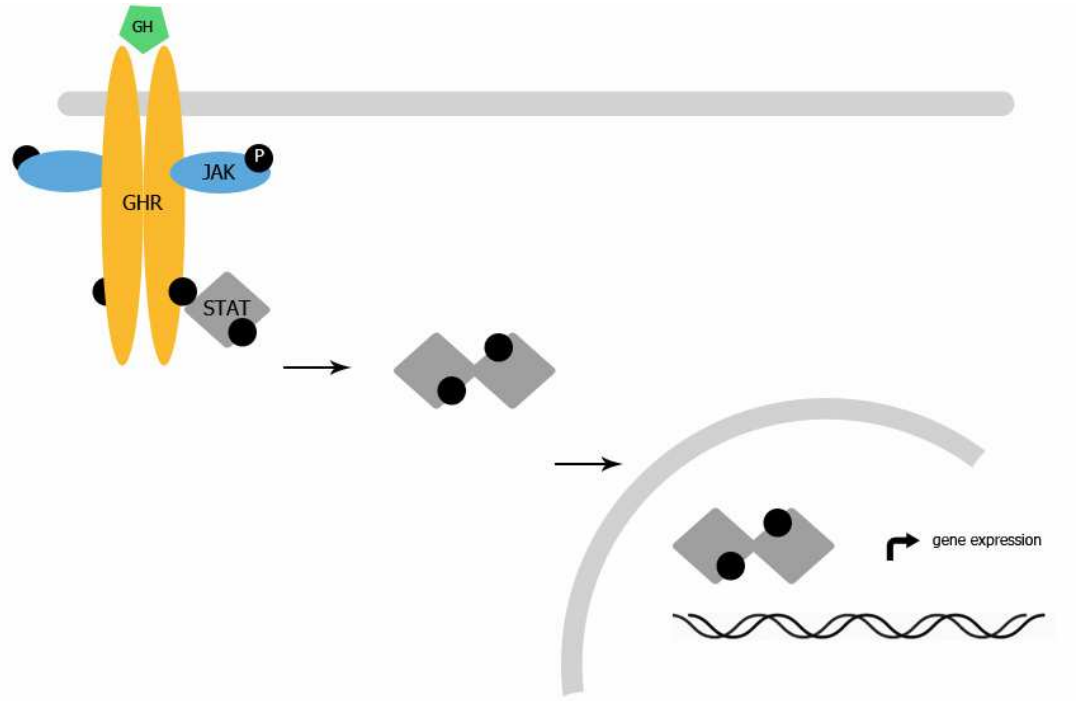

Figure 2. JAK/STAT signaling activated by GH. The scheme demonstrates the different players involved in JAK/STAT signaling pathway. GH: Growth Hormone; GHR: Growth Hormone Receptor; JAK: Janus Kinase; STAT: Signal Transducer and Activator of Transcription.

1.2.2.1. STAT 3

The 86 kDa protein STAT 3 becomes phosphorylated at tyrosine residue Y705 and is also able to form heterodimers with STAT1. This signal transducer was originally identified as an acute-phase response transcription factor initiated by interleukin-6 (IL-6); (Akira et al., 1994). Its main inducers comprise members of the IL-6 family, the IL-10 family, granulocyte-colony stimulating factor (G-CSF), Leptin, IL-21, IL-27, and different growth factors (Schindler and Plumlee, 2008). A lot of these signal factors are produced in tumors and lead to high STAT3 activity. In HCC the treatment and especially the targeted therapy is limited so far. Sorafenib blocks STAT3 activation in HCC as a multikinase inhibitor. It is of big interest to overcome sorafenib resistance, and also reach more specific interventions. Direct inhibition of STAT3 and blocking off

its DNA-binding sequence have shown great progress and already are in clinical trials. Another target depicts IL-6 and its receptor glycoprotein 130 (gp 130); (Subramaniam et al., 2013).

1.2.2.2. STAT 5

STAT5 a/b (STAT5a with a molecular weight of 94 kDa and STAT5b of 92 kDa) become phosphorylated at Y694/699 and are able to form homodimers or heterodimers with the coexisting STAT5 partner and other members of the STAT family, like STAT1 and STAT3 (Novak et al., 1996). The first description of STAT5 transcription factor was in response to prolactin and IL-3 stimulation (Wakao et al., 1994; Azam et al., 1995). Its main stimulants are IL-3 family members, GH, prolactin (Prl), thrombopoietin (Tpo), erythropoietin (Epo) and other cytokines (IL-2, IL-7, IL-9, IL-21); (Schindler and Plumlee, 2008). Except for the different preference of Prl and GH, STAT5a prefering Prl stimulation and STAT5b GH induction, the two isoforms are functionally overlapping.

In general STAT activation is fast and temporary, due to its strict negative regulation. Different families of proteins are involved in the regulation of STATs, like suppressor of cytokine signalling (SOCS), protein inhibitor of activated STAT (PIAS) and tyrosine phosphatases like the protein tyrosine phosphatase (PTP) family. The pathway can be inactivated by dephosphorylation of the receptors, the JAKs, directly at STAT proteins and by binding and occupying the DNA-binding site of STAT proteins (Kubo et al., 2003; Shuai and Liu, 2005).

1.2.3. PI3K/Akt/mTOR signaling

1.2.3.1. Akt

The serine/threonine protein kinase Akt is also known as Protein Kinase B (PKB). It depicts a key figure in regulation of growth, proliferation, survival, metabolism, migration and angiogenesis. The PI3K/Akt/mTOR signaling, in the following delineated in detail, can be activated through different triggers. Induction can be released via ligands like growth factors or insulin, via cytokines, via G-protein-coupled receptors and via stimulation by integrins (Foster et al., 2003; Wymann et al., 2003). Within the protein kinase superfamily, Akt is part of the AGC subfamily (protein

kinase A, G, C); (Manning et al., 2002). Three different isoforms, deriving from three different oncogenes, are known: Akt1, Akt2, Akt3 (PKBα, PKBβ, PKBɣ, respectively). Three functional domains reflect the conserved structure between the homologes. The N-terminal pleckstrin homology (PH) domain is necessary for binding to membrane lipids like PIP3 or to G proteins, secondly the functional central kinase domain is responsible for the catalytic activity and finally the C-terminal domain holds the hydrophobic motif (HM), a regulatory domain (Hanada et al., 2004). Studies with knockout mice revealed the different biological involvement of the individual isoforms. In this work Akt 2, molecular weight of 60 kDa, was assessed because of its relevance in insulin signaling and growth (Cho et al., 2001; Yang et al., 2004).

Ligand binding to the GHR leads to mutual autophosphorylation and activation of the receptor associated kinases JAK2, followed by phosphorylation of the receptor at tyrosine residues, creating binding sites for signaling molecules that lead to stimulation of class 1A Phosphoinositid-3-Kinases (PI3K). The PI3 kinases are connected to the receptor through adapter molecules like insulin receptor substrate (IRS) or through own regulatory subunits. Activated PI3K catalysis the phosphorylation of the membrane lipid phosphatidylinositol (4,5)-bis-phosphate (PIP2) to phosphatidylinositol (3,4,5) – tris-phosphate (PIP3). This step leads to recruitment of Akt from the cytosol to the plasma membrane, binding to PIP3. That interaction discloses phosphoinositide dependent protein kinase-1 (PDPK1) as well to access to PIP3 (Song et al., 2005). PDPK1 is responsible for the phosphorylation and partial activation of Akt at Thr308 in the activation loop (Alessi et al., 1997). Complete activation of Akt is not achieved before a second phosphorylation at Ser473 in the C-terminal regulatory domain by the mammalian Target of Rapamycin (mTOR) Complex 2 or by DNA-dependent protein kinase (DNA-PK); (Feng et al., 2004; Sarbassov et al., 2005).

One way of Akt leading to proliferation is achieved via phosphorylation of the cyclin/cyclin-dependant-kinases (CDK) inhibitors p21 and p27, which results in the inhibition of their capability to arrest the cell-cycle (Hanada et al., 2004). Amongst others transcriptional control of apoptosis is reached through inhibition of the Inhibitor of κ Binding (IκB) via the IκBα-Kinase-Komplex (IKK), which leads to the loss of inhibition of the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and expression of pro-survival genes. Glycogen synthase-Kinase 3 (GSK3) is another

target protein of Akt, in which through inhibition, the metabolic regulation of the apoptosis can be regulated (Song et al., 2005) (Figure 3).

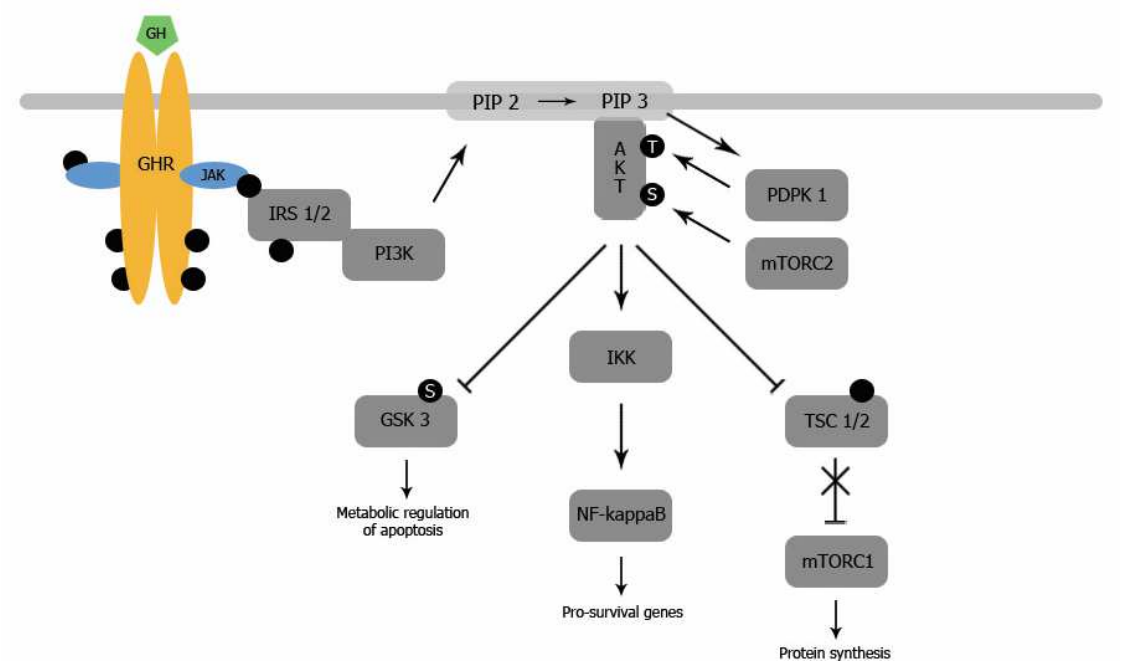

Figure 3. GH induced signaling via the PI3K/Akt signaling pathway including downstream components of Akt signaling. GH: Growth Hormone; GHR: Growth Hormone Receptor; JAK: member of the Janus Kinase family; IRS 1/2: Insulin Receptor Substrate; PI3K: class 1A Phosphoinositid-3-Kinases; PIP2: Phosphatidylinositol (4,5)-bis-phosphate; PIP3: Phosphatidylinositol (3,4,5) –tris-phosphate; PDPK 1: Phosphoinositide Dependent Protein Kinase-1; mTORC2: mammalian Target Of Rapamycin Complex 2; GSK 3: Glycogen Synthase Kinase 3; IKK: Inhibitor of κ Binding Kinase; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B-cells; TSC 1/2: Tuberous Sclerosis Complex 1/2; mTORC1: mammalian Target Of Rapamycin Complex 1.

The signaling cascade can be inactivated at several points. Dephosphorylation of PIP3 to PIP2 inhibits Akt indirectly. This conversion is regulated by the phosphatase and tensin homolog (PTEN) leading to PIP2 (4,5) or by the SH2-containing inositol polyphosphate 5-phosphatase (SHIP) leading to PIP2 (3,4); (Stambolic et al., 1998; Huber et al., 1999). Direct inhibition of Akt by dephosphorylation at Ser473 and/or at Thr308 is another way to regulate this serine/threonine kinase. This inhibition is regulated by different phosphatases like protein phosphatase 2A (PP2A) and PH domain and leucine-rich repeat protein phosphatase (PHLPPα); (Andjelkovic et al., 1996; Gao et al., 2005).

Increased activation of the Akt pathway due to failure of control mechanisms is associated with a range of malignancies. A failure in PTEN expression for example, is presumably associated to increased Akt activity (Romano 2013). In a study with 56 patients, HCC was related to increased Akt2 level in 38% (Xu et al., 2004). Furthermore, failure of Akt regulation is reported to be a critical modulator of increased resistance to chemo- or radiotherapy. On the other hand, inhibition of the PI3K/Akt/mTOR pathway can be avoided through signaling via the Raf/MEK/ERK pathway and therefore prepare chemoresistent cancer cell type (Romano 2013; Saini et al., 2013).

1.2.3.2. mTOR

mTOR is a 290 kDa enzyme, which belongs to the PI3K-related kinase family. It is involved in a broad range of biological processes such as metabolism, cell growth and aging. It is the target of the macrolide rapamycin (sirolimus), isolated from the bacteria Streptomyces hygrosopius. Rapamycin became of big interest in the transplant medicine as an immunosuppressant to prevent organ rejection. mTOR forms two different complexes, complex 1 (mTORC1) and complex 2 (mTORC2), which differ in their structure and function.

Complex 1 is activated via growth factors, energy input, cytokines, the canonical Wnt pathway and amino acids. Growth factors for example insulin or insulin like growth factor (IGF1), transmitting their signals via the PI3K/Akt and the Ras/Raf/Mek/Erk signaling pathways, interact with mTORC1 by inhibiting its upstream inhibitor, tumor suppressor tuberous sclerosis 1/2 complex (TSC1 - Hamartin; TSC2 - Tuberin), via phosphorylation. Down-regulation of mTORC1 is promoted through activation of the key regulator TSC 1/2 (Laplante and Sabatini 2012). A TSC 1/2-independent way of mTOR activation was also described in AKT signaling. Further downstream mTORC1 itself is involved in protein synthesis.

Much less is known about the second complex of mTOR, mTORC2. It is also reactive to growth factors like insulin but in contrast to mTORC1 it is insensitive to nutrients. As mentioned above Akt is one of the controling targets of mTORC2 within the AGC subfamily (Sarbassov et al., 2005; Laplante and Sabatini 2012).

The interest of mTOR in targeted therapy has risen precisely during the last years. Upregulation of the mTOR pathway can be observed in 40-50% of HCC. It is reported to be involved in sorafenib resistance and correlates with bad prognosis. In preclinical studies mTOR inhibitors have shown good results in tumor suppression. So far rapamycin and rapalogs (first generation mTOR inhibitors) are in clinical trial for advanced HCC and for adjuvant therpay in HCC patients after transplantation and TACE (Matter et al., 2013).

1.2.4. Downstream Akt: multiplayer GSK3-β

1.2.4.1. GSK3-β

The Ser/Thr Kinase GSK3-β was originally named after its primarily identification as the kinase of the glycogen synthase serving the regulation of the glycogenesis (Embi et al., 1980). Beyond the regulation of glycogenesis it is involved in proliferation, differentiation, survival and migration. Two isoforms are known, α and β. In this work the β-isoform is analyzed because of its more assembled role in relation to cell survival (Jope et al., 2007; Jacobs et al., 2012). GSK3-β is involved in two major pathways which are frequently dysregulated in liver cancer, PI3K/Akt and Wnt/β-catenin pathways (Jope et al., 2007; Llovet and Bruix, 2008). The regulation of GSK3-β by Insulin and growth factors occurs from its inhibition by phosphorylation at the N-terminal residue Ser9. Transmitted through the PI3K/Akt pathway, the phosphorylation at Ser9 is catalysed by PKB. The inhibition of GSK3 results, for instance, in dephosphorylation and activation of the glycogen synthase which leads to glycogenesis (Cross et al., 1995). The same residue of GSK3-β at Ser9 can be phosphorylated by other kinases of the mitogen-activated protein kinase (MAPK) cascade stimulated by growth factors. The epidermal growth factor (EGF) is also capaple of inhibiting GSK3 via the PI3K/Akt and the MAPK pathway (Shaw and Cohen, 1999). GSK3-β is mainly positive regulated indirectly through inhibition of upstream negative regulators (Jacobs et al., 2012).

GSK3 is principally located in the cytoplasm where it regulates most of its actions. However some target proteins or transcription factors are located in the mitochondrium and the nucleus, amongst others cyclin D1, c-Myc, c-Jun and NF-κB. In those compartments its activation is even higher than in the cytoplasm (Bijur and Jope, 2003).

The active GSK3 is regulated by binding proteins, with which it interacts forming protein complexes. One of the best investigated pathways in which GSK3 is involved is the Wnt/β-catenin pathway. Through polyphosphorylation of β-catenin GSK3 provokes its degradation via the proteasomal pathway (Jope and Johnson 2004) (Figure 4). The role of GSK3-β in apoptosis is bifunctional and very complex. Proapoptotic functions are described in direct p53 mediation, though there is conflicting data regarding p53 modulation (Watcharasit et al., 2002; Jacobs et al., 2012).

As mentioned above the connection between GSK3-β and the PI3K/Akt and Wnt/β-catenin pathways makes it an interesting player in carcinogenesis (Jope et al., 2007; Llovet and Bruix, 2008). As a crucial player in the inflammation process GSK3-β is not only involved in the pathogenesis of cancer, but also in Alzheimer´s disease, mood disorders, peripheral inflammation such as Arthritis and Diabetes. Administration of GSK3 inhibitor suppressed the proinflammatory response in mice precisely, opening a sight in therapeutic targeted therapy (Martin et al., 2005).

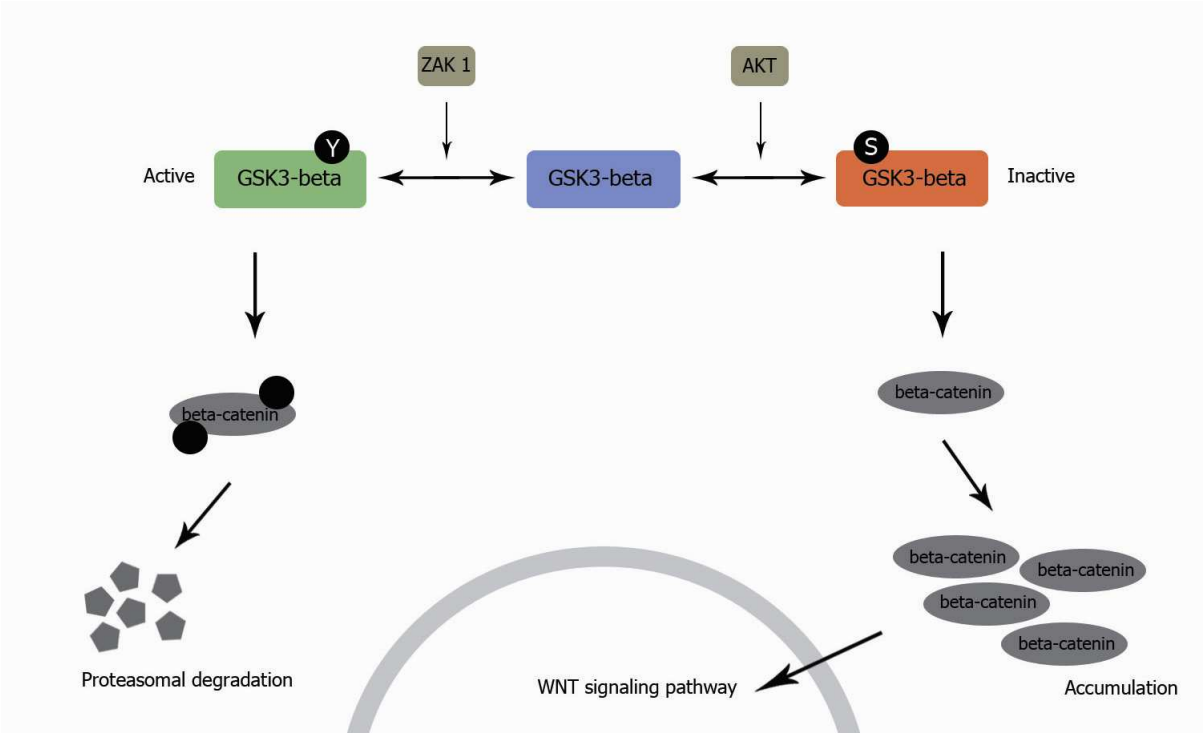

Figure 4. Regulation of GSK3-β via phosphorylation. Phosphorylation at Serine 9 (S) by Akt leads to inactivation of GSK3-β with stabilization and accumulation of β-catenin in the cytoplasm and subsequent transportation to the nucleus. Phosphorylation at Tyrosine 216 (Y) by ZAK1 (also known as AZK - Sterile alpha motif and leucine zipper containing kinase), amongst others, activates GSK3-β with subsequent degradation of β-catenin. Scheme is adapted from Jacobs et al., 2012

1.2.4.2. β-catenin

As a transcriptional coactivator β-catenin is a key player in the canonical Wnt signaling pathway. A lot of research has been done about its role in animal development indicating the importance in proliferation and differentiation (Nüsslein-Volhard and Wieschaus, 1980; De Robertis et al., 2000). The initial discovery as a member of the catenin family in Drosophila melanogaster (named armadillo as the homologous protein) revealed its relevance for cell-cell adhesion, making it just as interesting in cancer research (Nüsslein-Vollhard et al., 1984). Loosing cell-cell adhesion is a major step in development of malignancy (Perl et al., 1998). The transmembrane E-cadherin protein (epithelial calcium dependant adherin), which links cells to surrounded cells, is connected to α-catenin and the actin filament of the cytoskeleton via complexation with the cytoplasmic protein β-catenin. β-catenin belongs to the larger family of catenins consisting of α-catenin, β-catenin, ɣ-catenin/plakoglobin and δ-catenin (Kemler 1993). The loss of cell-cell adhesion is one of the characteristics in epithelial-mesenchymal transition (EMT), which cells go through building carcinomas.

The Wnt-signaling pathway is involved in the control of cytoplasmatic β-catenin. Members of the Wnt (Wingless – Int1) growth hormone family bind to the seven transmembrane domained Frizzled (Fz) receptor with its extracellular N-terminal cysteine-rich domains (CRD). The Fz receptor forms a heterodimer with the lipo-protein-related-protein 5/6 (LRP 5/6); (Bhanot et al., 1996; Dann et al., 2001). At the cytoplasmic fraction Fz is connected to Dishevelled (Dsh); (Chen et al., 2003). This interaction allows a second interaction between the coreceptor LRP5/6 and Axin, as well on the cytoplasmic area of the receptor. Without Axin, the degradation complex of β-catenin is incomplete, meaning inhibition of degradation and accumulation of β-catenin in the cytoplasm and the nucleus. In the nucleus β-catenin is able to form a complex with the transcription factor TCF/LEF (T-cell specific transcription factor/lymphoid enhancer-binding factor) leading to transcription control (Logan and Nusse, 2004, Clevers and Nusse, 2012).

Without any stimulus the scaffold protein axin binds GSK3, β-catenin, adenomatous-polyposis-coli protein (APC) and casein kinase I (CK1). In this constellation β-catenin is phosphorylated by CK1 and GSK3, preparing a recognition site for beta-transducin repeat-containing protein (β-TrCP), a component of the E3 ubiquitin ligase complex. β-

catenin then leaves the complex to get polyphosphorylated by β-TrCP (Clevers and Nusse, 2012). The last step leads to proteasome degradation. In the presence of stimulation of the Wnt/β-catenin pathway the axin-based complex is cleaved, followed by a decrease of β-catenin phosphorylation. This decrease leads to β-catenin accumulation, activation and transfer to the nucleus (Jope and Johnson 2004). A new model showed a different constellation of the degradation complex during activation via the Wnt-signaling pathway. Wnt binding induces the association of the intact degradation complex with the coreceptor LRP. Phosphorylated β-catenin stays trapped in the degradation complex. Only ubiquitination through β-TrCP is blocked, which inhibits proteasome degradation (Li et al., 2012).

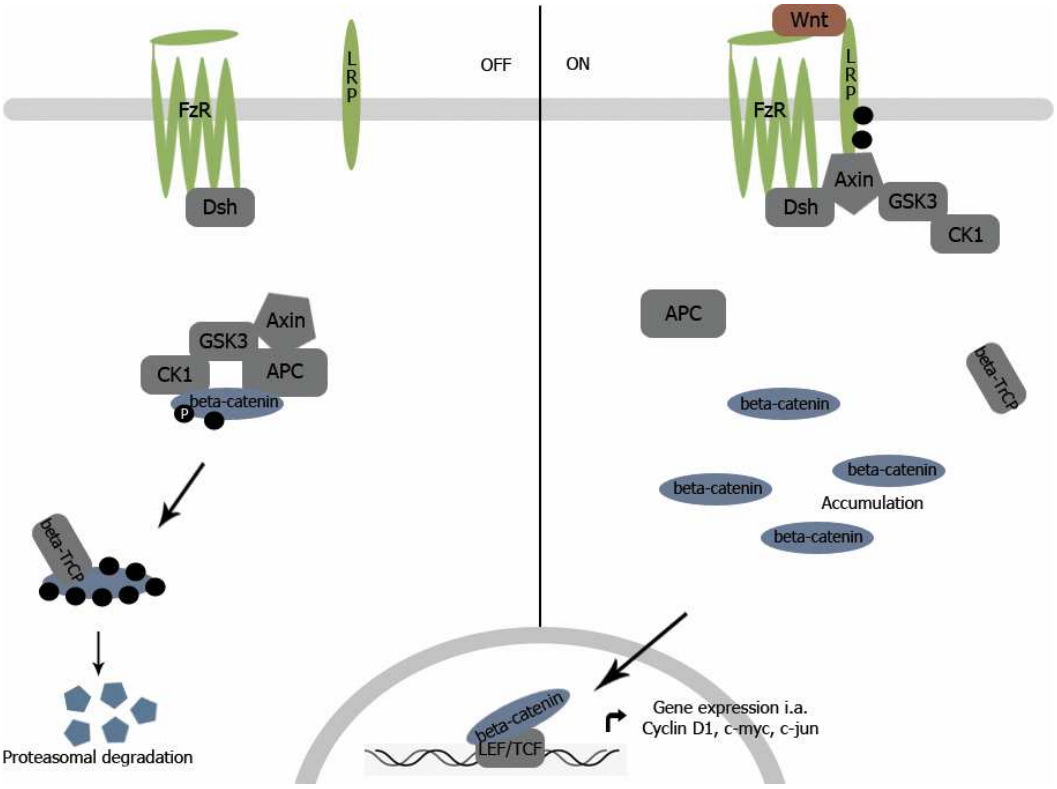

Figure 5. Wnt signaling pathway. On the left part of the scheme the ´off´ status, without extracellular stimulus, is demonstrated. Without stimulus of the Frizzled receptor (FzR), β-catenin is bound to its degradation complex in the cytoplasm with subsequent phosphorylation and proteasomal degradation. On the right part of the scheme the `on` status is demonstrated. As a result to extracellular ligand binding of Wnt (Wingless – Int1), axin binds to the phosphorylated coreceptor LRP 5/6 (Lipo-protein-related-protein 5/6), leading to inhibition of degradation of β-catenin, its accumulation in the cytoplasm and to transcription control after transportation to the nucleus. Dsh: Dishevelled; GSK-3: Glycogen Synthase Kinase-3; CK1: casein kinase I; APC: adenomatous-polyposis-coli protein; β-TrCP: beta-transducin

repeat-containing protein; TCF/LEF: T-cell specific transcription factor/lymphoid enhancer-binding factor. Scheme is adapted from Logan and Nusse, 2004; Clevers and Nusse, 2012.

The pattern of the expressed target genes induced by transcription complex β-catenin-TCF/LEF depends on the cell type. Amongst others are Cyclin D1, c-myc and c-jun (Logan and Nusse 2004; Clevers and Nusse 2012). Because of its role in cell-cell adhesion and in cell-cell signaling in development and differentiation β-catenin is involved in a variety of tumorigenesis. More common are mutations in upstream or downstream proteins of β-catenin. A common known mutation in a regulatory component is the early step mutation of APC in colorectal carcinoma (part of the adenoma-carcinoma-sequence). But also in HCC mutations of β-catenin itself were reported up to 26% out of 31 patients (de la Coste et al., 1998). In a more novel study activating mutations in the β-catenin encoded gene CTNNB1 were detected in about 28% out of 32 patients (Cieply et al., 2009).

1.2.5. NF-κB

The NF-κB/Rel family consists of five Rel proteins like NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65) and Rel B, forming homo- and heterodimers. The transcription factor RelA (p65 – indicating the molecular weight of 65 kDa) is integrated in an enormous range of processes and complex mechanisms. The focus in this work is on RelA (p65), the most common protein out of the larger family of NF-κB/Rel.

Firstly described in transcription of immunoglobulin light chain genes, further studies revealed the involvement NF-κB in immune and inflammatory responses, for example via lipopolysaccharide (LPS) and cytokines, antiviral response via interferon induction or directly adressing viruses through the NF-κB promoter binding site. In this context also physical stress conditions like ischemia, liver regeneration and hemorrhagic shock can induce NF-κB activity. The capability of activation of pro- and anti-apoptotic pathways by NF-κB should not be neglected. This indicates the elevation of cell survival followed to higher stress response (Sen and Baltimore 1986; Pahl 1999). The range of impact is extended by crosstalk to different transcription factors like STAT3, p53 and different kinases such as GSK3-β or PI3K (Hoesel and Schmid, 2013).

In general Rel/NF-κB transcription complexes gather in the cytoplasm bound to their inhibitors of the IκB-family (Inhibitor of κ Binding). Concrete stimulus like Tumor

Necrose Factor α (TNFα), Interleukin 1 (IL-1) or LPS induces rapid phosphorylation of IκB by the IκB Kinase (IKK), followed by polyubiquitination and degradation via the 26S proteasome pathway. This releases the NLS (nuclear localization signal), directing NF-κB to the nucleus (Traenckner et al., 1995; DiDonato et al., 1997). Downregulation and termination of NF-κB is described by translocation of new synthesized IκB to the nucleus and transportation of the transcription factor back into the cytoplasm via its nuclear export sequence (Arenzana-Seisdedos et al. 1997).

In a big range of cancers activated NF-κB can be detected, though in most cases it is not associated with genetic alterations, neither in NF-κB and IKK itself, nor in upstream components (Karin et al. 2002) supporting the thesis of a concrete connection between inflammation and cancer. In respect to hepatocarcinogenesis the effects of NF-κB have to be differentiated. Depending on the cell type, activation and inhibition of NF-κB show different results. In non-parenchymal cells activation of the transcription factor leads to inflammation, fibrosis and hepatocarcinogenesis. In contrast to that, in parenchymal cells inhibition NF-κB activation promotes hepatocarcinogenesis in some investigations (Luedde and Schwabe, 2011).

1.2.6. Oncoproteins

1.2.6.1. c-Myc

Initially it was described as the cellular homolog of the retroviral v-myc oncogene. B-myc, C-myc, L-myc, N-myc and s-myc compose the family of myc genes (Bishop, 1982; Cole, 1986). C-Myc targets an enormous variation of proteins involved in proliferation, growth and apoptosis. It interacts directly or indirectly (as a consequence of a direct target gene) with its target genes. Concerning DNA metabolism, c-Myc addresses for example DHFR (dihydrofolate reductase), p53 in relation to apoptosis and a number of cyclins in control of the cell cycle, just to name a few (Dang, 1999). Together with c-fos and c-jun it belongs to the early genes involved in the regulation of the priming phase of the cell cycle. In reference to the investigated cyclins in this work c-Myc is denoted to promote the activity of cyclinE/Cdk2 and cyclin D/Cdk4/6 complexes (Amati et al., 1998). A reciprocal relation between cyclin D and c-Myc is suggested.

In normal cells the expression of c-myc is strictly regulated by different players. Stimulus comes from cell cycle mediators itself, growth factors and contact to extracellular matrix (Gardner et al., 2002). Many tumors appear with elevated c-Myc levels. In comparison to the c-myc gene in normal cells, the gene found in tumors often remerges altered. Mainly found are chromosomal translocations, retroviral insertions and gene amplification, followed by constitutive activation (Kelly et al., 1986). But c-myc overexpression is not termless connected with oncogenic profile. The level of c-myc expression determines the cellular reaction (Murphy et al., 2008). Structural alterations of c-myc gene are also described for HCC in many cases (Llovet et al., 2008; Yuen et al., 2001). This makes c-Myc interesting for targeted therapy. In an inducible c-myc animal model, regression of carcinogenesis in HCC was reached by inactivation of c-myc and lead to differentiation of cells (Shachaf et al., 2004). Due to the complex mechanism and interactions of c-Myc in cellular proliferation, metabolism and apoptosis this basic approach is not available in clinics so far.

1.2.6.2. Activating-protein-1 (AP-1) complex

1.2.6.2.1. c-Fos

The transcription factor c-Fos, immediate early gene in the regulation of the cell cycle, is a member of the Fos protein family, consisting of c-Fos, Fos-B, Fos-B2, deltaFosB2, Fra-1 and Fra-2, serving the regulation of proliferation, differentiation and cell survival. C-Fos is a 340 amino acid protein (Barker et al., 1984), and represents the human homolog of the retroviral oncogene v-Fos (Van Beveren et al., 1983). After activation through external stimuli, the 55 kDa sized phospho-nucleoprotein, c-Fos heterodimerizes with a member of the Jun protein family (c-Jun, JunB, JunD) through its basic-region-leucine-zipper domain. This heterodimer forms the transcription factor AP-1 (activating-protein-1) complex, binding to TPA-responsive promoter elements (TREs) DNA regulatory regions (Sassone-Corsi et al., 1988; Angel and Karin, 1991). In contrast to members of the Jun protein family, Fos protein family members cannot form homodimers. However the c-Jun-c-Fos heterodimer binds 25 times more efficient to the AP-1 DNA binding site than the c-Jun homodimer form does (Halazonetis et al., 1988). Through external stimuli like growth factors and cytokines, the stimulus is transmitted and modulated by the JAK/STAT and MAPKinase pathway, amongst

others. The protein stability depends on the ubiquitin-26S proteosome pathway, controling the event of the AP-1 response, which is important for the regulation of cellular functions and the normal process of the cell cycle. Having half-lives of about 10 to 60 minutes, c-Fos and c-Jun are rapidly degragated (Tsurumi et al., 1995). Early studies revealed the transforming capability of c-Fos protein in cultured fibroblasts (Miller et al., 1984). Interestingly some studies preached a negative relation between the protooncogen c-fos and hepatocellular tumorigenesis in murine epithelial hepatocytes, leading to depolarization in association with inhibition of proliferation and even induction of cell death (Mikula et al., 2003). However, the majority is discussing the opposite fact. A clinical study described a significant difference of c-fos expression in favour of HCC tissue compared to non-tumoral tissue (Yuen et al., 2001). Recent studies indicated the positive influence of c-Fos protein in cell cycle regulation of hepatocytes, by a correlation of c-fos overexpression with high levels of inactive phosphorylated GSK3-β and consequential stabilized cyclin D1 within the nucleus (Güller et al., 2008).

1.2.6.2.2. c-Jun

C-Jun protein was first identified as the Fos-associated protein p39 (Haluska et al., 1987; Rauscher et al., 1988). Like all immediate early genes in cell cycle regulation c-jun is physiologically induced by growth factors and cytokines promoting proliferation or differentiation, therefore inhibiting apoptosis. On the other hand, cellular response to c-Jun activation can denote apoptosis itself. It depends on the cell type and the surrounding effects which direction is driven (Leppä et al., 1999). The investigation of the oncogenic potential of Jun homodimers through artificial stabilization led to enhanced activity of Jun in chicken embryonic fibroblasts (van Dam et al., 2001). Clinical study in patients with HCC showed high levels of c-Jun in both tumoral and non-tumoral tissue (Yuen et al., 2001).

1.2.6.3. Cell Cycle

The cell cycle is a highly controlled mechanism with checking points throughout the particular phases. It is divided into four parts: G1 (gap1), S-phase (synthesis), G2 (gap2) and finally the M-phase (mitosis). The focus in this work is put on cyclin D1 and

cyclin E as controling cyclins for the transition from G1- to S-phase. Cyclin A and cyclin B are in control of steps from S-phase to G2-phase and further on entering and leaving M-phase. Cyclins characteristicly are up- and downregulated during the cell cycle, depending on the interphase of cell division (Evans et al., 1993). All cyclins share the so called "cyclin box", which consists of 100 aminoacids and permits binding to the cyclin dependant kinases (CDKs) (Bobayashi et al., 1992; Noble et al., 1997). The cyclins are indispensable for the activation and regulation of the cyclin-dependant protein kinases. Together cyclins and CDKs form holoenzymes to pass restriction points of the cell cycle. As assembled complexes they enter the nucleus, where they get activated by a CDK-activating kinase (CAK); (Sherr et al., 1999). The level of the CDKs is more or less constant, while the cyclin level in contrast varies highly during cell cycle, as mentioned above.

1.2.6.3.1. Cyclin D1

Cyclin D1, the 35 kDa dominant member of the D-type cyclins (D1, D2 and D3), forms a complex with CDK4 or CDK6. It is induced during delayed early response to mitogenic growth factor stimulation via different transcription factors including AP-1, STATs, NF-κB, cAMP-response element (CRE) and T-cell factor (TCF); (Takahashi-Yanaga et al., 2007). Its expression lasts throughout the cell cycle. However, it only leads to interruption of the cell cycle when it is diminished before entering the S-phase (Sherr, 1995). As soon as the decision for cell division has been made, cells become unreceptive for growth-hormone signals (Pardee 1989). Because of its short half life of less than 30 minutes, phosphorylated at Threonine-286 by GSK3-β and further ubiquitination via the proteasomal pathway, continous stimulus is required to maintain its expression (Diehl et al., 1997). The activated complex cyclin D1/CDK4/6 partially phosphorylates Retinoblastom protein (pRB), which inhibits the E2F transcriptional activity. For the E2F release and activation, further phosphorylation of pRB, by the cyclin E/CDK2 complex is necessary. After complete phosphorylation and release of E2F, target genes for S-phase entry are expressed (Matsushime et al. 1994; Lavia et al. 1999).

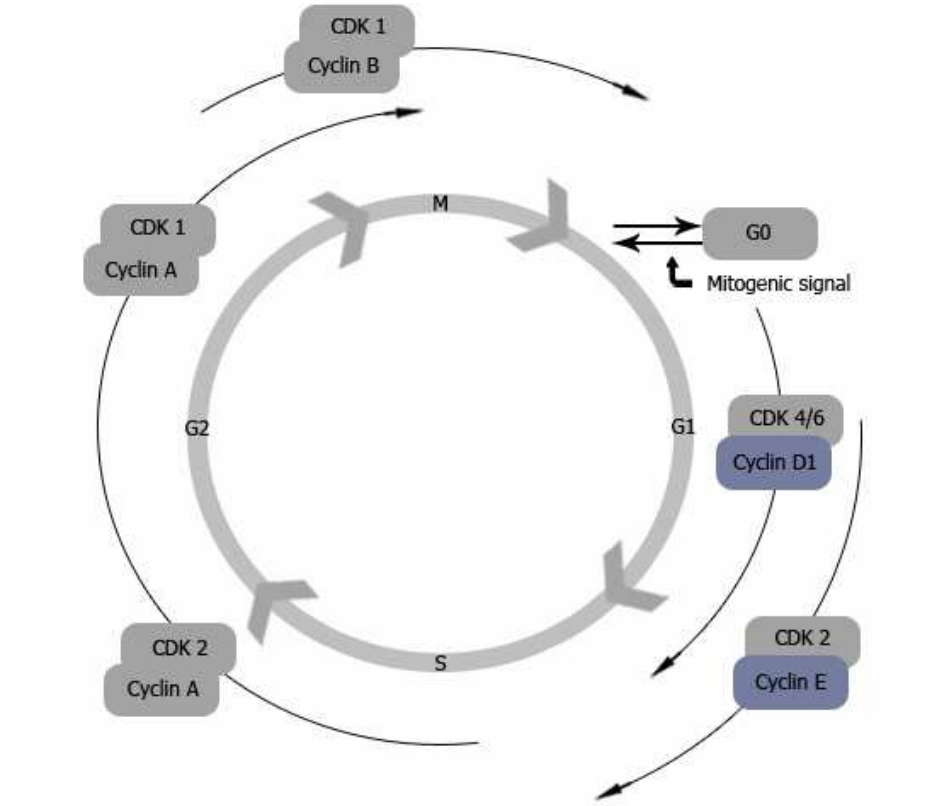

Figure 6. Cell cycle. The scheme demonstrates the four phases of the cell cycle: G1 (gap1), S-phase (synthesis), G2 (gap2) and M-phase (mitosis). Without mitogenic stimulation the cell cycle arrests in the G0 phase. Progression and passing of restriction points, from one phase to the following, is controlled by assembled complexes build of Cyclins and their dependant kinases (CDKs). Scheme is adapted from Takahashi-Yanaga et al., 2008.

1.2.6.3.2. Cyclin E

Like cyclin D1, cyclin E is a delayed early response gene in the cell cycle. It peaks at G1/S transition and builds a complex with and activates CDK2 (Lew et al., 1991). Via the activation of the transcription factor E2F it also triggers its own transcription (Duronio et al., 1995). Further negative and positive control of cyclin D1 and cyclin E expression by redistribution of the CDKs is regulated via p21 (Cyclin-dependent kinase inhibitor 1) and p27 (Cyclin-dependent kinase inhibitor 1B); (Sherr et al., 1999).

Cyclin D1 and cyclin E are associated with carcinogenesis. A lot of oncogenes are involved within the signal transduction leading to cyclin expression and cell division. Oncogenic mutations in these pathways more likely lead to increased E2F activity than the cyclin mutation itself (Takahashi-Yanaga et al., 2008; Stamatakos et al., 2010, Hwang et al. 2005). Using cyclin D1 as a therapeutic target in cancer is getting more and more into focus. Options consist in direct inhibition or in inhibition of its partner

kinases CDK4 & CDK6 (Musgrove et al., 2011). Another target is the modulator GSK3-β. Activation of the inhibitor of cyclin D1, GSK3-β, is expected to drive reduction in the cyclin D1 mRNA level (Takahashi-Yanaga et al., 2008).

2. Objective

The hypothesis of this work asserts that prolonged exposure of GH produces the exacerbation of signaling pathways associated with proliferation, growth and cellular survival and that the persistent alteration of these pathways in the liver lead to tumor development in mice. It is of big interest to elucidate the implicated molecular mechanisms in the development and progression of this oncogenesis. The general objective of this study was to elucidate the molecular pathogenesis and signal transduction pathways underlying the pro-oncogenic liver pathology induced by prolonged exposure to elevated GH level in mice.

Constitutive activation of some mediators like STAT3, Akt, mTOR, Erk1/2, EGFR and Src, which are involved in cell proliferation and survival, was shown in female young adult transgenic mice overexpressing GH (Miquet et al., 2008). In this work the attention is concentrated on downstream components and effectors of those signaling cascades and their involvement in the process of hepatotumorogenesis in the liver of GH-transgenic mice with preneoplastic and neoplastic pathology. The sexual dimorphic pulsatile GH secretion and the higher incidence of HCC in males, both in humans and in mice (Rogers et al., 2007; Ruggiere et al., 2010), bundled the focus in this work on sexual dimorphism within molecular alterations in preneoplastic lesions in young adult GH-transgenic mice compared to their normal controls.

The liver expression and activation of signaling mediators involved in cell growth, proliferation and survival will be determined by immunoblotting and immunhistochemsitry in order to establish the relation between the molecular alterations and tumor development. The proposed study will contribute to determine the mechanisms by which GH promotes higher incidence of tumors and to establish safer conditions for the therapeutic use of growth hormone.

3. Materials and Methods

3.1 Materials

3.1.1. Reagents and kits

Table 1. Reagents and kits

material	company
acrylamide	Bio-Rad Laboratories, Inc.; USA
Amersham Hyperfilm ECL	GE Healthcare; USA
aprotinin	Sigma-Aldrich; USA
BCA Protein Assay Reagent	Pierce Biotechnology, Inc.; USA
bisacrylamide	Bio-Rad Laboratories, Inc.; USA
bromophenol blue	Sigma-Aldrich; USA
BSA	Sigma-Aldrich; USA
Coomasie Blue	Sigma-Aldrich; USA
ECL Plus Western Blotting Detection System	GE Healthcare; USA
EDTA	Sigma-Aldrich; USA
glicerol	Merck; USA
glycine	Sigma-Aldrich; USA
HEPES	Sigma-Aldrich; USA
Molecular weight markers for polyacrylamide gel electrophoresis	Bio-Rad Laboratories, Inc.; USA
Peroxidase substrate kit, DAB sk-4100	Vector Laboratories, Inc.; USA
PMSF	Sigma-Aldrich; USA
PVDF membranes	GE Healthcare; USA
R.T.U. VECTASTAIN Universal Quick Kit	Vector Laboratories, Inc.; USA
SDS	Sigma-Aldrich; USA
sodium fluoride	Sigma-Aldrich; USA
sodium pyrophosphate	Sigma-Aldrich; USA
sodium vanadate	Sigma-Aldrich; USA
Tris HCl	Sigma-Aldrich; USA
Triton X-100	Sigma-Aldrich; USA
Tween 20	Sigma-Aldrich; USA
2-ME	Sigma-Aldrich; USA

3.1.2. Primary Antibodies

Table 2. Primary Antibodies

antibody	source	dilution	company
anti-PCNA	rabbit	1:1000	Santa Cruz Biotechnology Laboratories
anti-phospho-STAT5 Tyr694/696	mouse	1:1000	Upstate Laboratories
anti-STAT5	rabbit	1:4000	Santa Cruz Biotechnology Laboratories
anti-phospho-STAT3 Tyr705	rabbit	1:500	Cell Signaling Technology Inc.
anti-STAT3	mouse	1:1500	Transduction Laboratories
anti-phospho-Akt Ser473	rabbit	1:500	Cell Signaling Technology Inc.
anti-Akt2	rabbit	1:2000	Cell Signaling Technology Inc.
anti-phospho-mTOR Ser2448	rabbit	1:500	Cell Signaling Technology Inc.
anti-mTOR	rabbit	1:500	Cell Signaling Technology Inc.
anti-phospho-GSK3-ß Ser9	rabbit	1:1000	Cell Signaling Technology Inc.
anti-GSK3-ß	rabbit	1:2000	Cell Signaling Technology Inc.
anti-ß-catenin (WB)	rabbit	1:1000	Cell Signaling Technology Inc.
anti-ß-catenin (IHQ)	rabbit	1:50	Santa Cruz Biotechnology Laboratories
anti-phospho-NF-kB p65 Ser536	rabbit	1:500	Cell Signaling Technology Inc.
anti-NF-kB p65	rabbit	1:1000	Cell Signaling Technology Inc.
anti-c-Fos	rabbit	1:500	Cell Signaling Technology Inc.
anti-c-Jun	rabbit	1:500	Cell Signaling Technology Inc.
anti-c-Myc	rabbit	1:500	Cell Signaling Technology Inc.

anti-cyclin E	rabbit	1:2000	Abcam Inc.
anti-cyclin D1	rabbit	1:200 (IHQ) 1:1000 (WB)	Santa Cruz Biotechnology Laboratories
anti-ß-actin	rabbit	1:1000	Sigma-Aldrich
anti-ß-tubulin	rabbit	1:10000	Abcam Inc.

3.1.3. Secondary Antibodies

Table 3. Secondary Antibodies

antibody	source	dilution	company
Anti-rabbit-IgG-HRP	goat	1:10000	Santa Cruz Biotechnology Laboratories
Anti-mouse-IgG-HRP	horse	1:10000	Santa Cruz Biotechnology Laboratories

3.2. Animal Model

Young adult, 9weeks old, transgenic mice overexpressing GH were studied as well as old animals, more than one year of age. All the experiments were performed in PEPCK-bGH transgenic mice. PEPCK-bGH mice containing the bovine GH (bGH) gene fused to control sequences of the rat phosphoenolpyruvatecarboxykinase (PEPCK) gene (McGrane et al. 1990) were derived from animals kindly provided by Dr. TE Wagner and Dr. JS Yun (Ohio University, Athens, OH, USA). The hemizygous transgenic mice were derived from a founder male and were produced by mating transgenic males with normal C57BL/6 x C3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Matings produced approximately equal proportion of transgenic and normal progeny. Normal siblings of transgenic mice were used as controls. In 9 weeks old animals, females and males were used. In advanced ages, more than one year of age, only females were included in the study. The mice

were housed 3-5 per cage in a room with controlled light (12 h light/day) and temperature (22° ± 2° C). The animals had free access to food (Lab Diet Formula 5001; PMI Inc., St. Louis, MO, USA) and tap water. The appropriateness of the experimental procedure, the required number of animals used, and the method of acquisition were in compliance with federal and local laws, and with institutional regulations.

3.3. Sample Processing

3.3.1. Solubilization of liver extracts

Mice were killed by cervical dislocation under isofluorane anesthesia, the livers were removed and stored at -70˚C until analysis. A portion of the liver was homogenized in solubilization buffer at 4˚C in a ratio of 10 ml of buffer per gram of tissue weight. The solubilization buffer was composed of 1% Triton, 100 mM Hepes, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.035 trypsin inhibitory units/ml aprotinin at pH 7.4. The resultant homogenates were centrifuged at 100,000 xg for 40 minutes at 4˚C. Protein concentration of supernatants was determined by the BCA assay. The solubilized samples were diluted in solubilization buffer into a concentration of 8 mg/ml, following a dilution with Laemmli buffer 2x (Laemmli, 1970), to achieve a final protein concentration of 4 mg/ml. Finally the diluted samples were boiled for 5 minutes and stored at –20 °C until electrophoresis.

3.3.2 Microscopic analysis

For microscopic analysis, freshly dissected livers were fixed in 10% formalin, dehydrated and embedded in paraffin to process sections at a thickness of 5 μm. For immunostaining and histological staining, liver sections were deparaffinized with two incubations of 5 minutes in xilol, and rehydrated in EtOH 100% (2x5 min) and EtOH 96% (2x5 min), followed by two incubations of H_2Od, each 5 minutes of duration.

3.3.2.1 Histology

For histomorphological evaluation, hematoxylin and eosin (H&E) staining was performed using standard procedures.

3.3.2.2 Immunohistochemical Staining

Deparaffinization and rehydration of liver sections was performed as described above. Because of possible alterations in the threedimensional structure of the proteins during embedded fixation, which could lead to loss of immunological reaction of some of the antigens, liver sections were subjected to antigenic recuperation with sodium citrate 0,01M, pH6,0 for 30 minutes at 98˚C. After antigenic recuperation, slides were cooled at room temperature in the citrate buffer for 20 minutes and then washed twice in H_2Od for 5 minutes. For inhibition of the endogenous peroxidase, recuperated liver sections were incubated with hydrogen peroxide 3% in PBS for 30 minutes at room temperature. This step was followed by a 10 minutes incubation in Triton X-100 0,3 % in PBS for better penetration of antibodies. To prevent nonspecific binding of antibodies, the sections were first blocked with bovine serum albumin (BSA) 1% w/v in PBS for 1 hour and secondly blocked with horse serum 2,5% (R.T.U. VECTASTAIN Universal Quick Kit) for 2 hours at room temperature.

Incubation with primary antibodies anti-PCNA (1:1000), anti-cyclin D1 (1:200) or anti-β−catenin (1:50) diluted in PBS containing BSA 1% was performed overnight at 4 ºC. Negative controls were performed in parallel in slides in which the primary antibody was replaced by PBS with BSA 1%. Subsequently, incubations with biotin-labeled secondary antibodies (anti-mouse/rabbit/goat; PAN-specific Ab) followed by incubation with streptavidin-horse radish peroxidase complex were each performed for 30 minutes at room temperature (R.T.U. VECTASTAIN Universal Quick Kit). Throughout the protocol, washing of the liver sections was perfomed with PBS between the mentioned steps. The antigen-antibody binding was visualized with diaminobenzidine (DAB) (Peroxidase substrate kit, DAB sk-4100), which produces a brown coloured precipitate in the presence of the peroxidase, insoluble in alcohol and xilol. After two washings in H_2Od for 5 minutes each, sections were counterstained with hematoxylin. Finally liver sections were dehydrated through two 1 minute incubations with EtOH 96%, two 3 minutes incubations with EtOH 100% and 2 incubations of xilol for each 5 minutes.

H&E and immunostained sections were observed under light microscopy using a Leica DM2000 microscope, photomicrographs were obtained using a Leica DFC400 digital camera and Leica Application Suite software (Leica Microsystems). Measurements were carried out using the image analyzer Image J 1.45s software. The cell and nuclear size were estimated by counting the number of hepatocytes per field and the nuclear area, respectively. Only cells that were unequivocally parenchymal in origin were analyzed. Hepatocyte proliferation was determined by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Ten fields per animal were analyzed, and all hepatocytes within the field were counted. Hepatocyte proliferation was expressed as the percentage of PCNA positive (brown stained) nuclei.

3.3.3. Western blotting

Samples were subjected to electrophoresis in SDS-polyacrylamide gels using Bio-Rad Mini Protean apparatus (Bio-Rad Laboratories). Equal amount of total protein (40µg) was loaded in each lane. For differentiation between resulting size distributed bands an amount of 10µg of molecular weight markers (Bio-Rad) was added in the first lane of each gel. Electrotransference of proteins from gel to polyvinylidenedifluoride membranes was performed for 1 hour at 100 mA per transferred membrane (constant current) using the V20-SDB semi-dry blotting apparatus (Scie-Plas) in 0.025 M Tris, 0.192 M glycine, 20% (v/v) methanol and 0.03% (w/v) SDS (pH 8.3). To reduce non-specific antibody binding, membranes were incubated for 1 hour at room temperature in T-TBS buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 0.02% w/v Tween 20, pH 7.6) containing 3% w/v BSA. The membranes were then incubated overnight at 4 °C with the primary antibody. Antibodies were diluted in T-TBS buffer containing 0.3% BSA. After washing with T-TBS, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase for 1 hour at room temperature and washed in T-TBS. Immunoreactive proteins were revealed on detection films (Hyperfilm) by enhanced chemiluminescence (ECL-Plus, Amersham Biosciences). After all detection steps membranes were stained with Coomasie Blue to control the total protein load and blotting efficiency. Membranes were washed in T-TBS buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 0.02% w/v Tween 20, pH 7.6) and afterwards stained with 0.1% w/v Coomassie in methanol/water, 1:1 for one minute. Subsequent destaining was

performed in acetic acid/ethanol/water, 1:5:4 for 20 minuntes (Welinder and Ekblad, 2010).

3.4. Analysis

3.4.1. Statistical analysis

Experiments were performed analyzing all groups of animals in parallel, n representing the number of different individuals used in each group. Results are presented as mean ± SEM of the number of samples indicated. Band intensities were quantified by digital densitometry using Gel-Pro Analyzer 4.1 software (Media Cybernetics, USA). Statistical analyses were performed by One Way ANOVA followed by the Newman-Keuls multiple comparison test using the GraphPad Prism 4 statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Student's t test was used when the values of two groups were analyzed. Data were considered significantly different if $P < 0.05$.

4. Results

4.1. Liver weight, histological characteristics & hepatocellular proliferation

4.1.1. Liver weight, histological characteristics & hepatocellular proliferation in young adult mice

Young adult, 9 weeks old, PEPCK-bGH-transgenic mice develop hepatomegaly, with a higher liver weight than normal mice, still explicit after correction by body weight. Those findings are in accordance with previous publications using GH-overexpressing transgenic mouse models (Orian et al., 1989; Wanke et al., 1991; Hoeflich et al., 2001). No sex differences are detected in the relative liver weight, either in normal or in transgenic mice (Table 4). No macroscopic liver lesions can be observed at this age. The microscopic analysis show preneoplastic morphological alterations in young adult GH-transgenic mice. Areas of dysplasia are observed in both sexes. Cellular and nuclear size is enlarged with nuclear polymorphism, predominantly in centrolobular areas (Figure 7A-B). To evaluate hepatocyte hypertrophy, the relative cell size was estimated by counting hepatocytes per field in liver sections. Hepatocyte enlargement is indicated by a significant decrease in the number of hepatocytes per microscopic field in transgenic mice in comparison to normal controls. In both genotypes males present a significantly lower number of hepatocytes per field than females (Figure 7C). Transgenic mice also display significantly higher mean nuclear size than their normal controls (Figure 7D) with a shift to larger nuclear areas and a broader area range in the size distribution profile (Figure 7E-G). The nuclear area is bigger in males than in females in both genotypes (Figure 7D). The two types of graphs concerning the size distribution profile provide different perception on the nuclear area range. The line graph (Fig. 7E) denotes a continous fluent view, in which single peaks inbetween the nuclear area range can be observed and allows to get better insight into the wide range of the distribution profile. The bar graph (Fig. 7F&G) combines nuclear area ranges to focus the mean distribution. To evaluate hepatocellular proliferation, immunohistochemistry and immunoblotting analysis for the S-phase related proliferating cell nuclear antigen (PCNA) was performed. Transgenic mice display a marked increase in the percentage of hepatocytes positive for nuclear labeling with PCNA compared to their normal controls, with males presenting significantly more

PCNA positive cells than females in the transgenic group (Figure 8A). Similar results are observed by immunoblotting analysis, although the differences are less pronounced (Figure 8B). Normal males exhibit a consistent trend to lower PCNA levels than normal females, though this is not statistically significant (Figure 8A-B). GH-transgenic mice display higher content of β-actin and β-tubulin, proteins that are usually used when checking uniformity of loading in immunoblotting assays (Figure 8C). Changes in β-actin and β-tubulin were described in preneoplastic and tumoral cells, which accompany the structural reorganization of the cytoskeleton (Miller et al., 2008; Popow et al., 2006). Consequentially, loading homogeneity was controled by immunoblotting with anti-STAT5, as no differences in the amount of this protein are found between adult normal and GH-transgenic animals (Figure 8C), in accordance with previous reports (Miquet et al., 2004; Miquet et al., 2008; Sotelo et al., 2008). At last PVDF membranes were further stained with Coomasie Blue to assess homogeneity of protein loading (Figure 8C).

Table 4. Body and liver weight in 9 weeks old mice overexpressing GH and normal controls.

	Body weight (g)	**Liver weight (g)**	**Liver wt/Body wt (%)**
Normal females	20.1 ± 0.6 (a)	0.91 ± 0.03 (a)	4.5 ± 0.1 (a)
Normal males	24.2 ± 0.8 (b)	1.10 ± 0.06 (a)	4.5 ± 0.1 (a)
GH-Tg females	36.5 ± 0.9 (c)	2.73 ± 0.09 (b)	7.5 ± 0.1 (b)
GH-Tg males	38.4 ± 1.0 (c)	2.8 ± 0.1 (b)	7.5 ± 0.1 (b)

Data are presented as mean value ± SEM (n = 9). Different letters denote significant differences at $p < 0{,}001$.

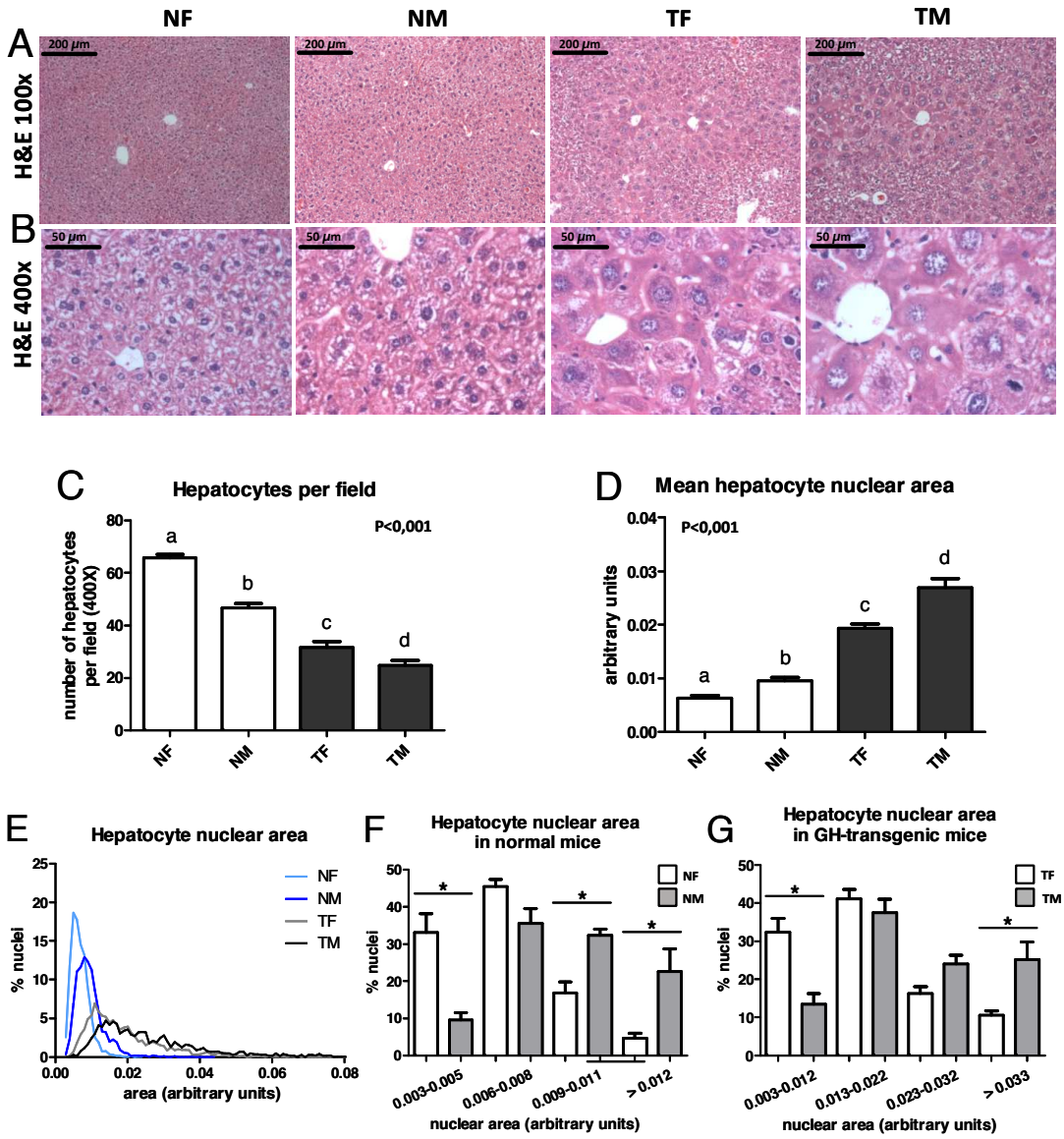

Figure 7. Histological analysis of livers from young adult GH-overexpressing transgenic mice and normal controls. Representative photomicrographs of liver sections from normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice are shown. (A) H&E staining, original magnification 100x; (B) H&E staining, original magnification 400x; (C) Analysis of hepatocytes number per visual field (original magnification 400x). The analysis of the hepatocyte nuclear area is displayed in (D) as mean hepatocyte nuclear area; (C and D) Groups denoted by different letters are significantly different. (E, F and G) Hepatocyte nuclear area size distribution for 9 weeks old normal and GH-transgenic mice. Data are the mean ± SEM of nine sets of different individuals per group. (F and G) Asterisks indicate significant differences between males and females for a same nuclear area range at $p < 0.05$.

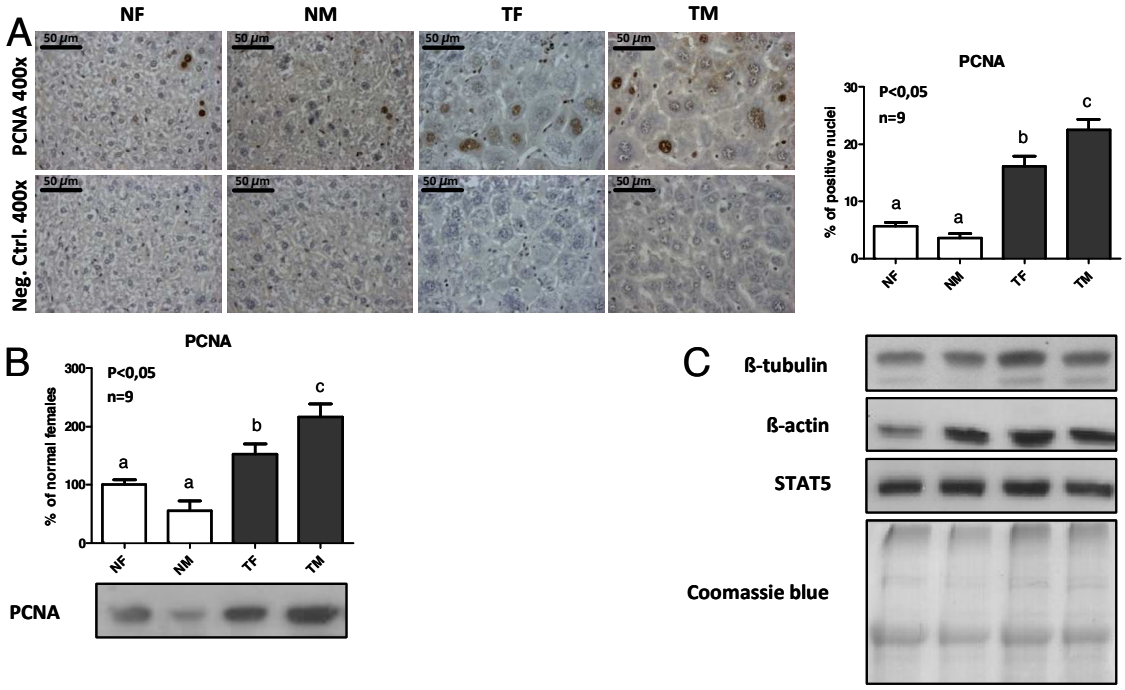

Figure 8. PCNA protein content and immunostaining in young adult mice (A, B and C). To evaluate hepatocellular proliferation, the expression of the proliferating cell nuclear antigen (PCNA) was assessed in the liver from young adult, 9 weeks old, normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice. (A) Immunohistochemical staining of liver sections with anti-PCNA antibody: representative microphotographs are shown; the % of positive hepatocyte nuclei was determined in 10 high-power fields per mouse, nine animals per group were analyzed. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody. (B) Liver extracts were analyzed by immunobloting with anti-PCNA antibody. Quantification was performed by scanning densitometry and expressed as % of the mean values in normal female mice. Data are the mean ± SEM of nine sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. A representative result of immunoblots is shown. (C) Loading controls were performed by immunoblotting with anti-β-tubulin, anti-β-actin and anti-STAT5 antibodies and by staining the PVDF membrane with Coomasie blue. Each lane contains 40 µg of solubilized liver proteins from an individual animal.

4.1.2. Liver weight, histological characteristics & hepatocellular proliferation in old mice

The data for old PEPCK-bGH-transgenic mice, more than 1 year old, concerning body and liver weight (Table 5), is similar to the described changes in 9 weeks old mice. The difference between hepatomegalic and normal liver weight is even more outstanding. Noticeably, body weight does not differ significantly between old GH transgenic mice and their normal controls. This is based on the fact that old GH-transgenic mice become significantly leaner than normal controls. GH favours lean body composition by activating lipolysis, therefore reducing adipose tissue, and by promoting protein addition in muscle (Palmer et al., 2009). In contrast to young adult mice, macroscopic liver lesions emerge in form of fibrotic nodules. Microscopically cellular and nuclear size is enlarged in GH-transgenic mice with nuclear polymorphism, in accordance to results obtained for young adult transgenic mice, more concentrated in centrolobular areas (Figure 9A-B). According to the evaluation of hepatocyte hypertrophy, transgenic mice reveal a significant decrease in hepatocytes per microscopic field in comparison to normal controls. The number of hepatocytes per field in tumoral areas of transgenic mice decrease significantly less than those from non-tumoral areas (Figure 9C). The hepatocyte mean nuclear area in the tumoral region is significantly smaller than in the non-tumoral tissue from transgenic mice and significantly bigger than the nuclear area of normal controls (Figure 9D). The shift to larger nuclear areas and to a broader area range in the size distribution profile is even more dominant in old transgenic mice compared to normal controls than in 9 weeks old mice (Figure 9E-F). Tumoral and non-tumoral areas reveal a complete inhomogenous nuclear area range with the tumoral area demonstrating an even bigger amount of peaks, indicating the higher variety of individual nuclear area in tumoral regions (Figure 9E-F). Immunohistochemistry and immunoblotting analysis of PCNA for hepatocellular proliferation evaluation is not as homogeneous as shown in young adult mice. Immunohistochemistry for PCNA reveals a marked rise in proliferation for transgenic tissue in comparison to normal controls (Figure 10A). Similar results are observed by immunoblotting analysis, although the differences are less explicit. In immunohistochemistry and immunoblotting analysis proliferation rates compared between tumoral and non-tumoral tissue in GH-transgenic

mice are not significantly different (Figure 10A-B). In old animals STAT5 was choosed as loading control in Western blot for the same reason like for young adult animals.

Table 5. Body and liver weight in old female mice overexpressing GH and normal controls.

	Body weight (g)	Liver weight (g)	Liver wt/Body wt (%)
Normal	41.4 ± 3.8 (a)	1.5 ± 0.2 (a)	3.7 ± 0.3 (a)
GH-Tg	47.1 ± 3.8 (a)	4.1 ± 0.5 (b)	8.6 ± 0.5 (b)

Data are presented as mean value ± SEM (n = 8). Different letters denote significant differences at $p < 0{,}001$.

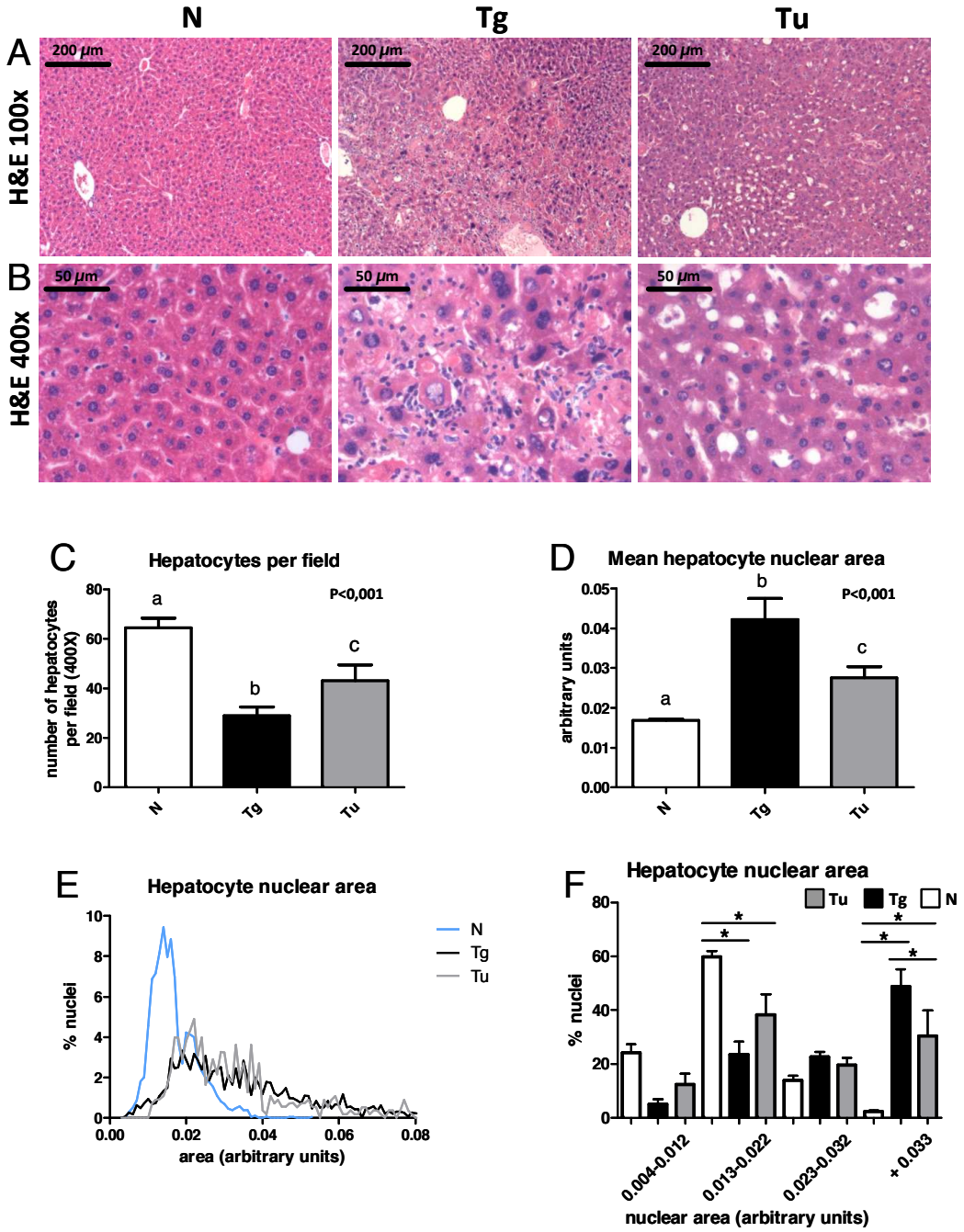

Figure 9. Histological analysis of livers from old, female GH-overexpressing transgenic mice and normal controls. Representative photomicrographs of liver sections from normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu) are shown. (A) H&E staining, original magnification 100x; (B) H&E staining, original magnification 400x; (C) Analysis of hepatocytes number per visual field (original magnification 400x). The analysis of the hepatocyte nuclear area is displayed in (D) as mean hepatocyte nuclear area; (C and D) Groups denoted by different letters are significantly different. (E and F). Data are the mean ± SEM of eight sets of different individuals per group. (F) Asterisks indicate significant differences between the tumoral and non-tumoral region in transgenic mice and normal controls for a same nuclear area range at $p < 0.05$.

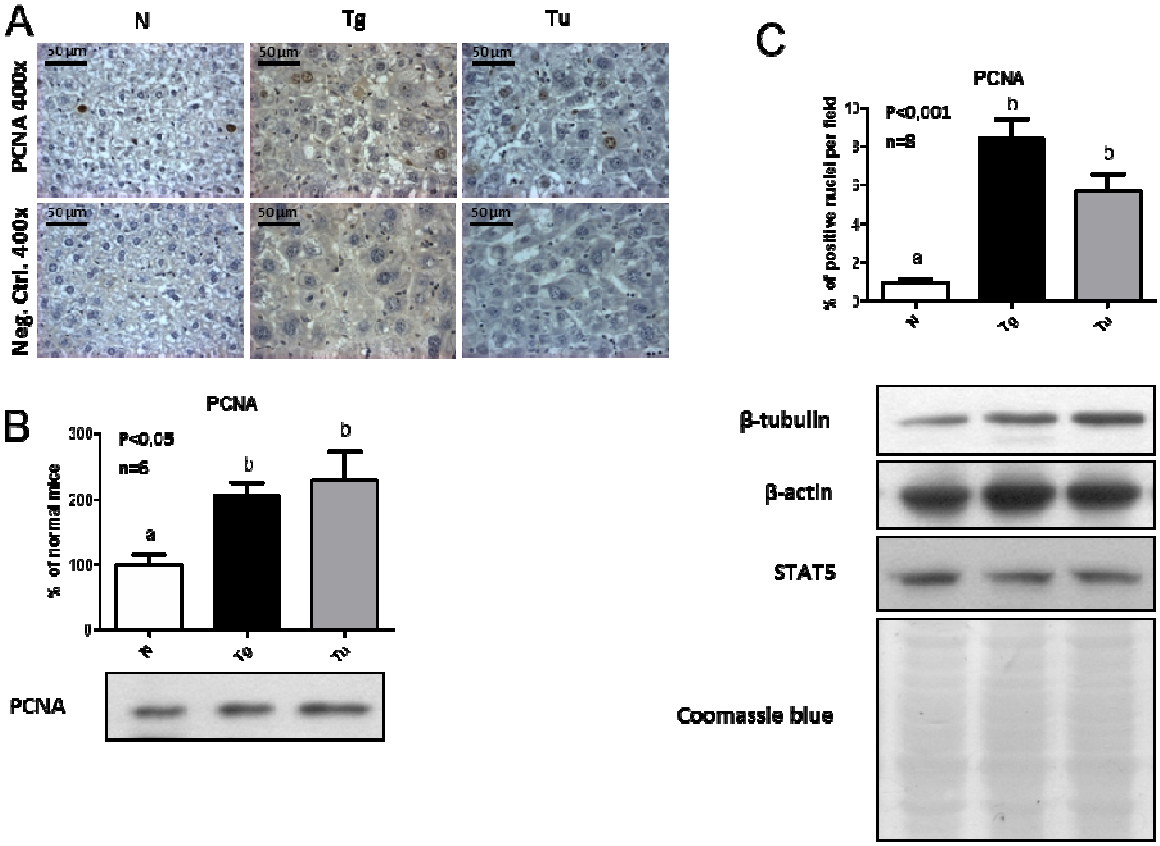

Figure 10. PCNA protein content and immunostaining in old, female mice (A, B and C). To evaluate hepatocellular proliferation, the expression of the proliferating cell nuclear antigen (PCNA) was assessed in the liver from old female, more than 1 year of age, normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu). (A) Immunohistochemical staining of liver sections with anti-PCNA antibody: representative microphotographs are shown; the % of positive hepatocyte nuclei was determined in 10 high-power fields per mouse, eight animals per group were analyzed. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody. (B) Liver extracts were analyzed by immunobloting with anti-PCNA antibody. Quantification was performed by scanning densitometry and expressed as % of the mean values in normal female mice. Data are the mean ± SEM of eight sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. A representative result of immunoblots is shown. (C) Loading controls were performed by immunoblotting with anti-β-tubulin, anti-β-actin and anti-STAT5 antibodies and by staining the PVDF membrane with Coomasie blue. Each lane contains 40 µg of solubilized liver proteins from an individual animal.

For each protein, the phosphorylation and protein content was determined by immunoblotting and the phosphorylation/protein content ratio was calculated.

4.2. Expression and activation of STAT3, STAT5, Akt2 & mTOR

4.2.1. Expression and activation of STAT3, STAT5, Akt2 & mTOR in young adult mice

Previous reports showed that female transgenic mice (4-6 months old) exhibit higher hepatic basal phosphorylation of STAT3 and protein overexpression of Akt and mTOR, among other signaling mediators. In contrast STAT5 protein content does not exhibit overexpression in GH-transgenic mice compared to normal controls (Miquet et al., 2008). In the present thesis the protein expression and activation of these signaling mediators was assessed in 9 weeks old female and male mice in parallel, to analyze if the molecular alterations described present sexual dimorphism. 9 weeks old GH-transgenic mice do not reveal any sex differences for the analyzed proteins (Figure 11A-D). STAT5 and Akt, though, present higher phosphorylation level in male young adult normal mice compared with normal female controls. The observed elevation for those two mediators indicates an increase in the relative activation of these proteins, as the higher phosphorylation levels are not related to elevated protein expression compared to normal females (Figure 11A&C). Transgenic mice from both sexes display basal phosphorylation levels of STAT5 which are not statistically different from their corresponding normal controls (Figure 11A).The high levels of activated Akt observed in transgenic mice seem to be a consequence of elevated protein levels (Figure 11C). Both sexes of transgenic mice show significantly higher phosphorylation levels of STAT3 at its activating residue, Tyr705, compared to normal mice. STAT3 protein levels are slightly elevated in transgenic mice, but this difference is not statistically significant from normal controls (Figure 11B). Transgenic mice also exhibit higher phosphorylation of mTOR at Ser2448 in both sexes, which is most probably the result of elevated abundance of this protein.

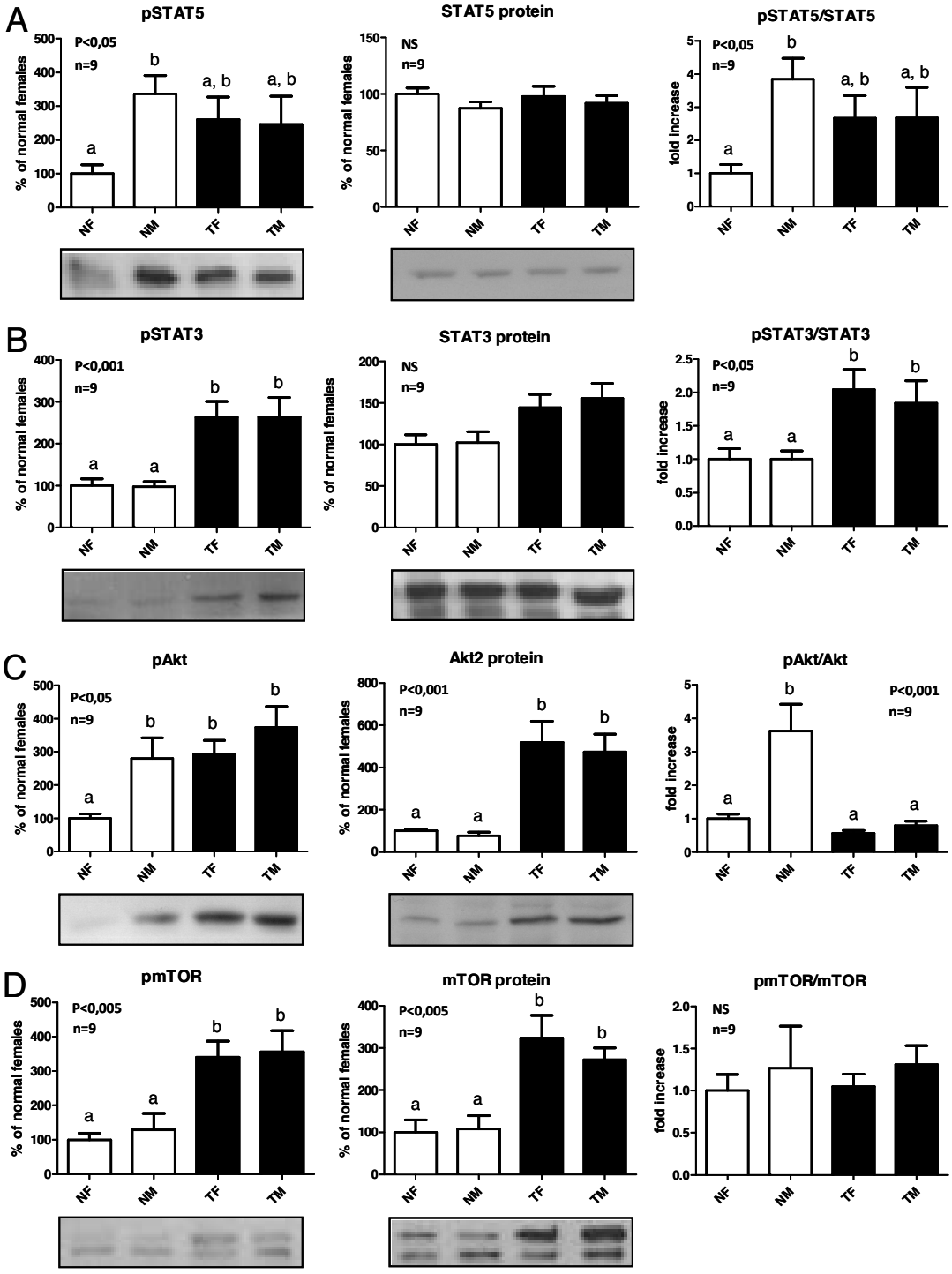

Figure 11. Expression and phosphorylation of STAT5, STAT3, Akt and mTOR in the liver of young adult GH-transgenic mice and normal controls. Liver extracts from young adult normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice were analyzed by immunoblotting to determine the phosphorylation and protein content. (A) STAT5 phosphorylation at Tyr694/696 (pSTAT5), protein content and phosphorylation/protein content ratio. STAT5 was also used as a control for equal loading. (B) STAT3 phosphorylation at Tyr705 (pSTAT3), protein content and phosphorylation/protein content ratio. (C) Akt phosphorylation at Ser473 (pAkt), Akt2 protein content, phosphorylation/protein content ratio. (D) mTOR phosphorylation at Ser2448 (pmTOR), protein content, phosphorylation/protein content ratio. Results are expressed as % of the mean values in normal female

mice. Data are the mean ± SEM of nine sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant.

4.2.2. Expression and activation of STAT3, STAT5, Akt2 & mTOR in old mice

As these proteins may participate in the hepatocarcinogenesis process, it was of interest to evaluate if the progress of the hepatic pathology that is observed with age, is accompanied by further changes in the signaling mediators in study. For that purpose, the expression and activation of STAT5, STAT3, Akt and mTOR was also assessed in old female GH-transgenic mice and normal controls. In accordance to previous findings concerning STAT5 (Miquet et al., 2008) no overexpression or higher activation is observed in old GH-transgenic mice. Old normal mice reveal a marginal elevation in activation of STAT5 compared to tissue of transgenic animals, though no statistical significant difference is observed (Figure 12A). Tumoral and non-tumoral tissue in transgenic mice do not show any difference for protein content or activation for STAT5 (Figure 12A). STAT3 denotes an elevation in phosphorylation level in old transgenic mice in relation to protein content, indicating an increase of relative activation of the protein, with non-tumoral tissue of transgenic mice presenting statistical significant difference, in contrast to tumoral tissue (Figure 12B). Activated Akt level in old GH-transgenic mice is significantly elevated in non-tumoral areas of transgenic mice compared to normal siblings (Figure 12C). Phosphorylated Akt level in tumoral zones as well is elevated compared to normal controls, but reveals less activated, not significantly different, phospho-Akt content than in non-tumoral areas (Figure 12C). The difference of activated Akt protein content for tumoral zones is not significantly different either in relation to normal controls, or to non-tumoral zones. Those findings might be due to higher protein level for Akt2 in non-tumoral zones. Eventually the relative activation of Akt in transgenic animals is less augmented in reference to normal siblings (Figure 12C). Elevation of mTOR activation is observed for old GH-transgenic mice compared to normal controls with a tendency to higher phosphorylated mTOR level in tumoral tissue than in non-tumoral zones (Figure 12D). The difference between tumoral and non-tumoral tissue does not differ statistically. The elevated phosphorylation of mTOR appears to be a consequence of elevated protein content, as

no statistical significant variation can be reported in the phosphorylation/protein content ratio (Figure 12D).

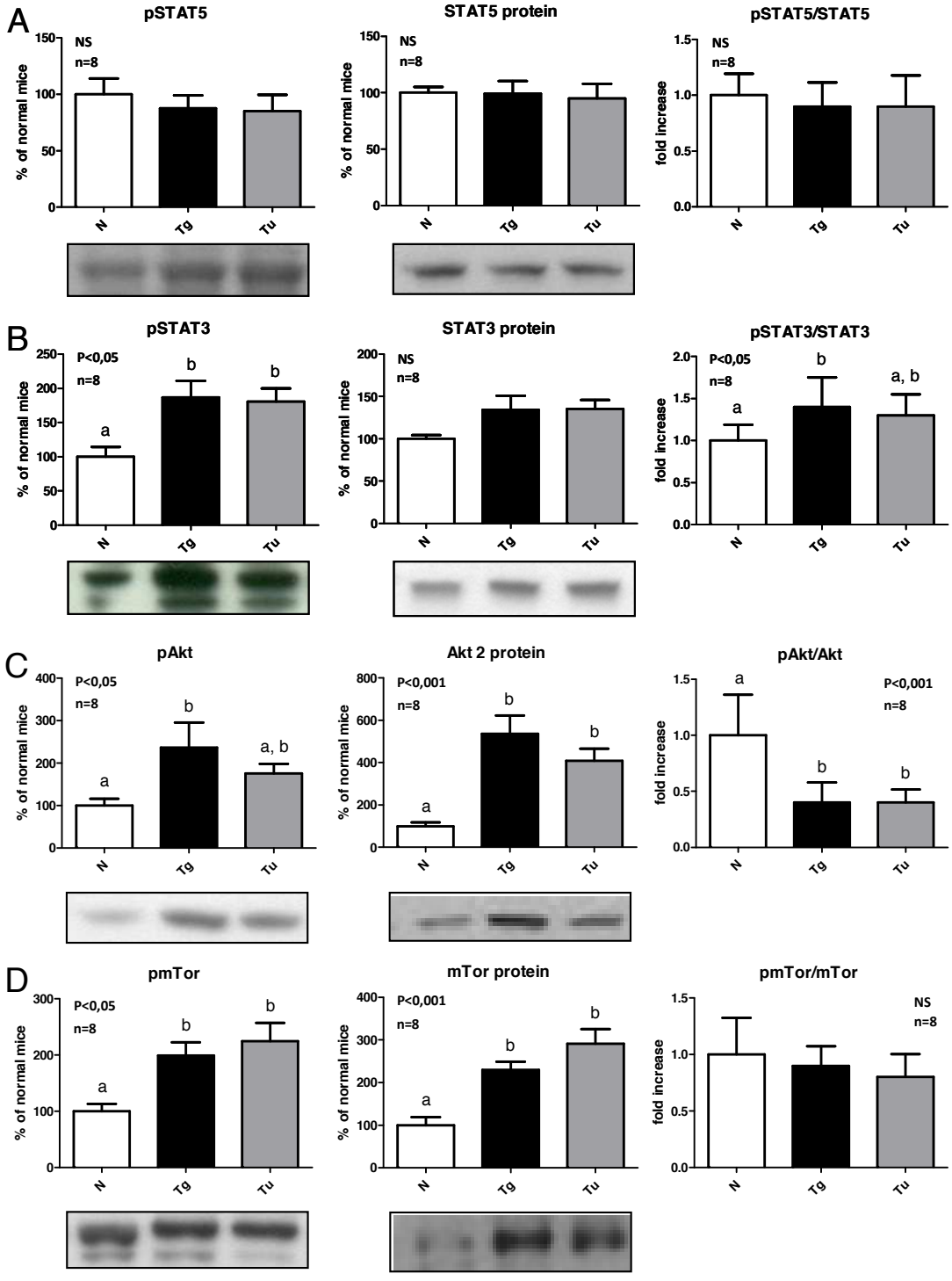

Figure 12. Expression and phosphorylation of STAT5, STAT3, Akt and mTOR in the liver of old, female GH-transgenic mice and normal controls. Liver extracts from old normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu) were analyzed by immunoblotting to determine the phosphorylation and protein content. (A) STAT5 phosphorylation at Tyr694/696

(pSTAT5), protein content and phosphorylation/protein content ratio. STAT5 was also used as a control for equal loading. (B) STAT3 phosphorylation at Tyr705 (pSTAT3), protein content and phosphorylation/protein content ratio. (C) Akt phosphorylation at Ser473 (pAkt), Akt2 protein content, phosphorylation/protein content ratio. (D) mTOR phosphorylation at Ser2448 (pmTOR), protein content, phosphorylation/protein content ratio. Results are expressed as % of the mean values in normal female mice. Data are the mean ± SEM of eight sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant.

4.3. Expression of GSK3-β and β-catenin

Akt and other Ser/Thr kinases phosphorylate the GSK3-β isoform at Ser9, inhibiting its activity, and consequently stabilizing cyclin D1 and β-catenin protein levels (Jope et al., 2007; Jacobs et al., 2012; Nicholson and Anderson, 2002; Takahashi-Yanaga and Sasaguri, 2008). The accumulation of β-catenin in the cytoplasm promotes its translocation to the nucleus, where it activates the transcription of several oncogenes, including cyclin D1, c-Myc and c-Jun (Jope et al., 2007; Nicholson and Anderson, 2002; Takahashi-Yanaga and Sasaguri, 2008). In 9 weeks old normal mice a higher inhibition of activation of GSK3-β through phosphorylation at Ser9 is observed in males compared to their female siblings. In the protein content, only a very small non statistical significant difference can be seen between normal female and male mice (Figure 13A). These findings are in accordance with the higher activity of Akt in males (Figure 11C). In transgenic mice no sex differences can be detected (Figure 13A). GH-transgenic animals reveal elevated GSK3-β protein content in males and females compared with normal controls. The phosphorylation levels of the transgenic animals display an even higher increase in comparison to normal controls, even when corrected by protein content. In old mice inhibition of activation of GSK3-β at Ser9 is significantly elevated in non-tumoral tissue in GH-transgenic animals in relation to normal controls. This might be a consequence of the likewise risen GSK3-β protein content of non-tumoral tissue (Figure 14A). The tumoral zones of GH-transgenic mice expose less inhibition of activation and do not show significant difference, neither to non-tumoral area nor to normal controls. Protein content of GSK3-β in tumoral zones equals the content in non-tumoral zones, indicating a significant decrease of relative protein inhibition of activation in tumoral tissue (Figure 14A).

The protein content of β-catenin is increased in male and female 9 weeks old GH-transgenic mice (Figure 13B). Immunohistochemistry analysis indicates a stronger staining in transgenic mice compared to normal controls. While β-catenin in normal

mice is only weakly marked in the cell membrane area, in GH-transgenic animals the protein is located in the cell membrane area and spread in the hepatocyte cytoplasm (Figure 13C). Protein content of β-catenin is significantly increased in old, more than 1 year old GH-transgenic mice, both in tumoral and non-tumoral tissue (Figure 14B). Findings in immunohistochemistry in old animals show strong cytoplasmatic and clear membrane staining. In contrast to 9 weeks old animals staining in old GH-transgenic mice includes some of the nuclei, in both tumoral and non-tumoral areas. As in 9 weeks old female mice β-catenin can be hardly detected in normal controls at advanced ages (Figure 14C).

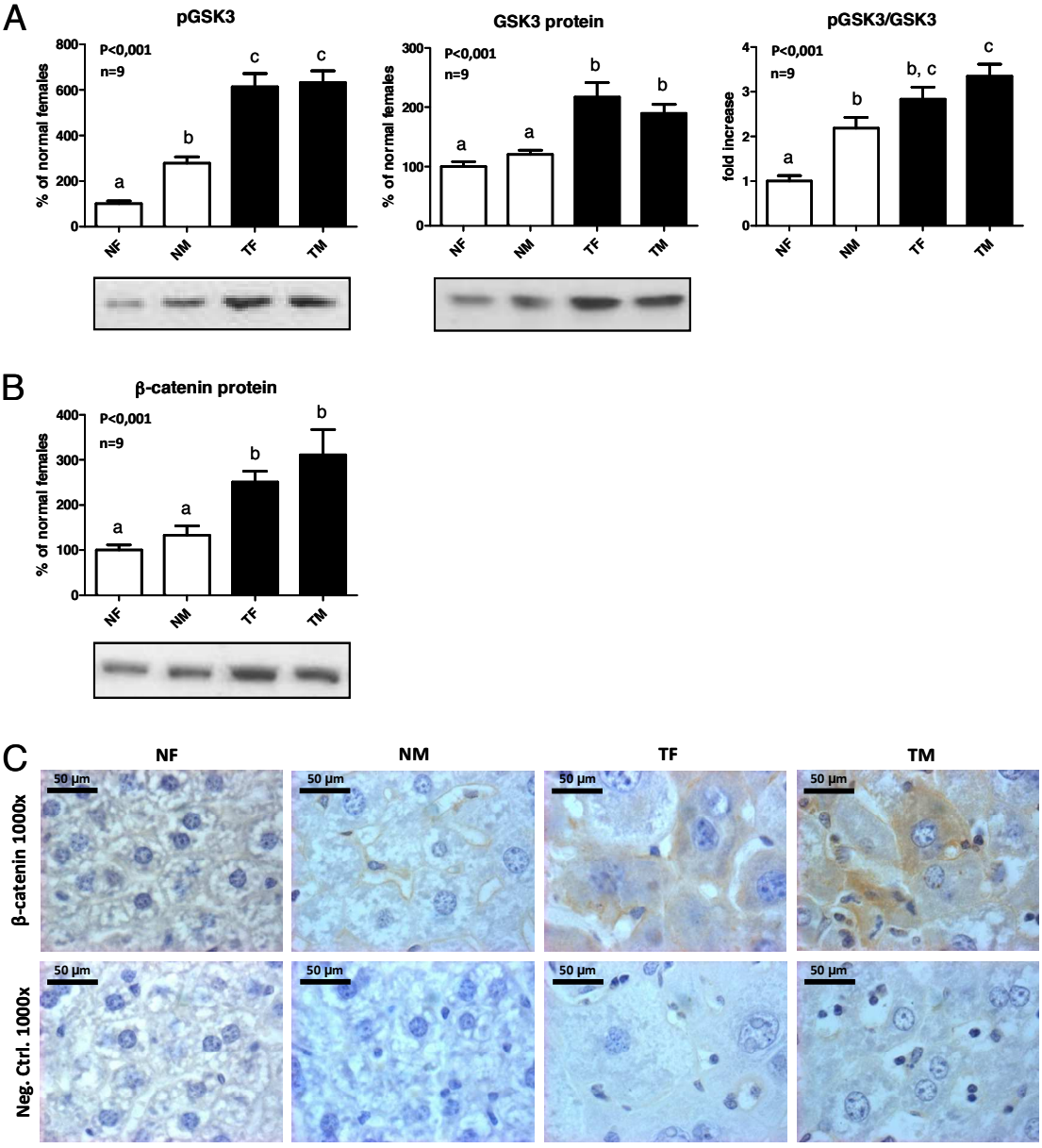

Figure 13. Expression of GSK3-β and β-catenin in the liver of young adult GH-transgenic mice and normal controls. Liver extracts from young adult normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice were analyzed by immunoblotting to determine the phosphorylation and protein content. (A) Phosphorylation of GSK3-β at Ser9 (pGSK3β), GSK3-β protein content and phosphorylated GSK3-β levels corrected by GSK3-β protein content. (B) β-catenin protein content. Results are expressed as % of the mean values in normal female mice. Data are the mean ± SEM of nine sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS: not significant. (C) Immunohistochemical staining of liver sections with anti-β-catenin: representative microphotographs are shown. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody.

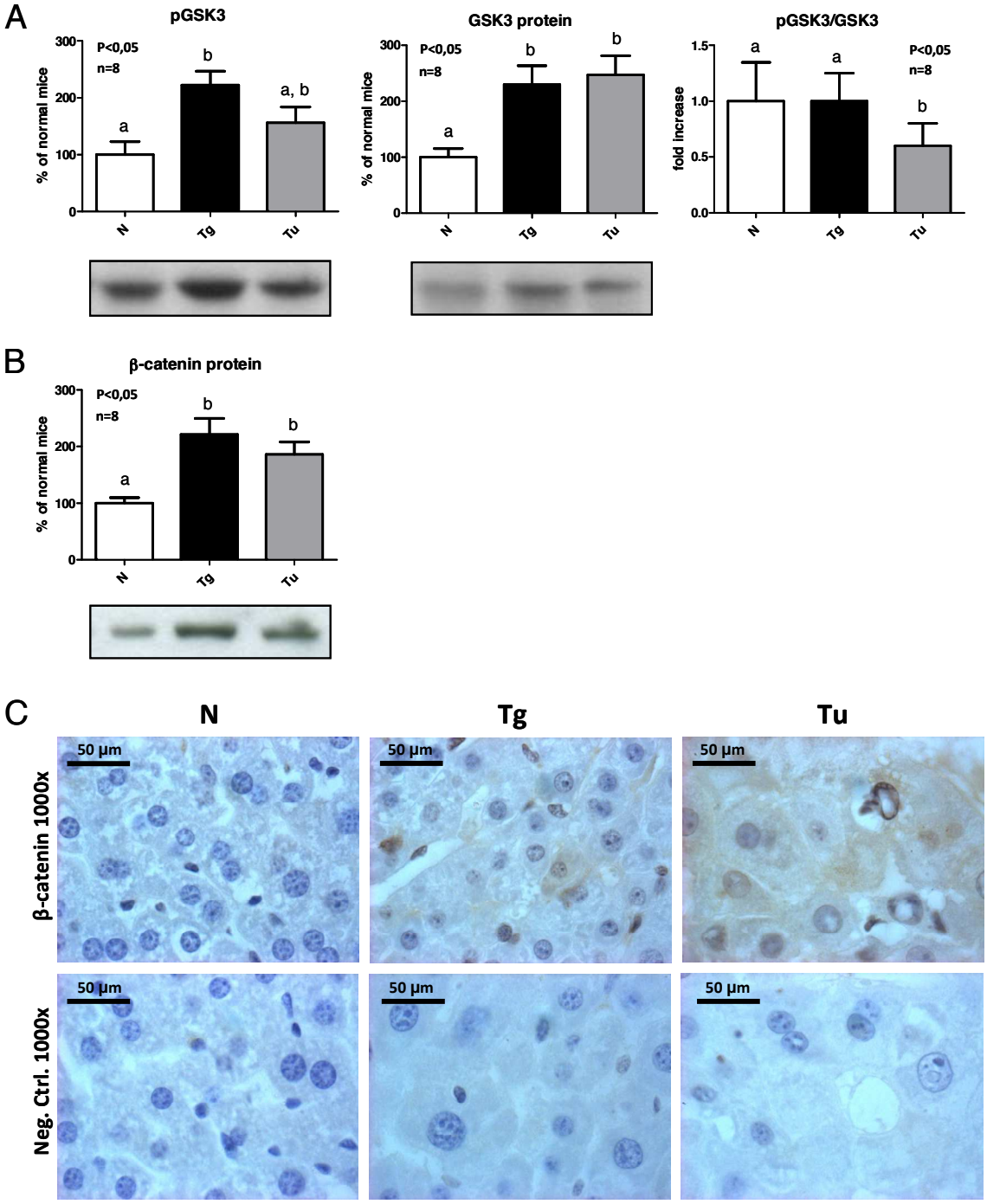

Figure 14. Expression of GSK3-β and β-catenin in the liver of old, female GH-transgenic mice and normal controls. Liver extracts from old normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu) were analyzed by immunoblotting to determine the phosphorylation and protein content. (A) Phosphorylation of GSK3-β at Ser9 (pGSK3-β), GSK3-β protein content and phosphorylated GSK3-β levels corrected by GSK3-β protein content. (B) β-catenin protein content. Results are expressed as % of the mean values in normal female mice. Data are the mean ± SEM of eight sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS: not significant. (C) Immunohistochemical staining of liver sections with anti-β-catenin: representative microphotographs are shown. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody.

4.4. Phosphorylation and expression of NF-κB

The member of the Nuclear Factor κB family p65 NF-κB (RelA) shows significantly higher protein content in young adult transgenic mice. In parallel the phosphorylated protein increases significantly comparing GH-transgenic mice and normal siblings. No sex differences can be detected, either in protein content or in the activation pattern (Figure 15A). The results for old mice indicate the same increase for GH-transgenic mice. Tumoral zones, though, show a tendency to higher expression of the protein NF-κB than non-tumoral tissue does, without statistical significance. The activation and the protein content of NF-κB indicate significant increase for transgenic mice in relation to normal controls (Figure 15B).

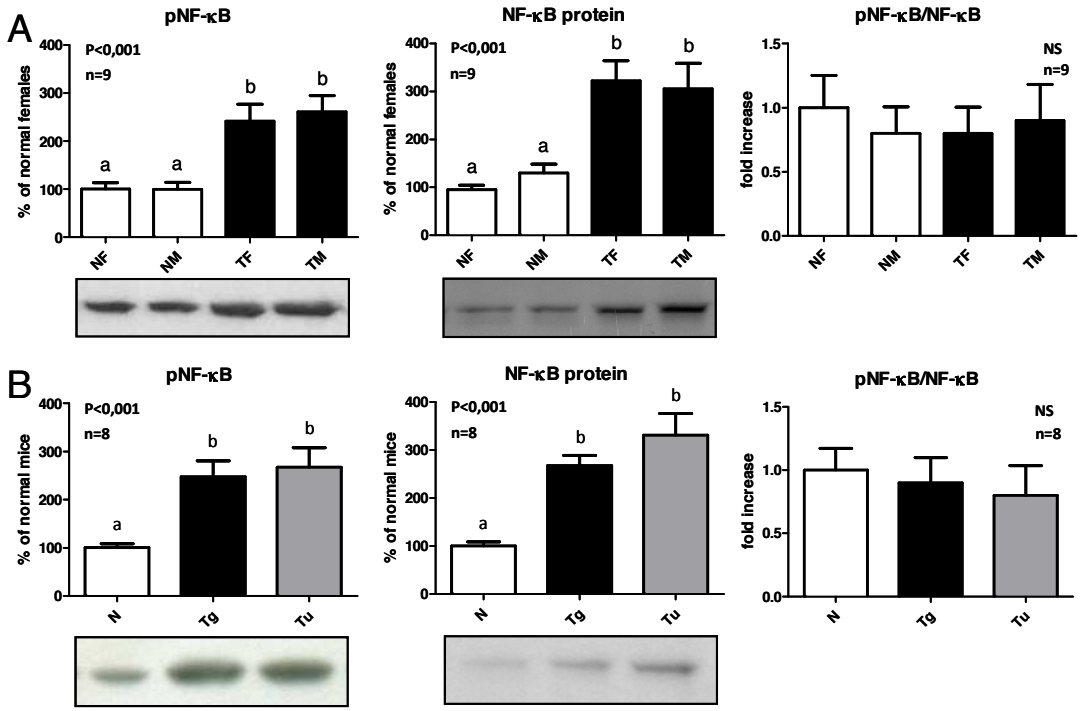

Figure 15. Expression and phosphorylation of NF-κB p65 (RelA) in the liver of young adult GH-transgenic mice and normal controls (A) and in the liver of old, female GH-transgenic mice and normal controls (B). (A) Liver extracts from young adult normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice were analyzed by immunoblotting to determine the phosphorylation and protein content. NF-κB p65 (Rel A) phosphorylation at Ser536 (pNF-κB), protein content, phosphorylation/protein content ratio. Results are expressed as % of the mean values in normal female mice. Data are the mean ± SEM of nine sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant. (B) Liver extracts from old normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu) were analyzed by immunoblotting to determine the phosphorylation and protein content. NF-κB p65 (Rel A) phosphorylation at Ser536 (pNF-κB), protein content, phosphorylation/protein content ratio. Results are expressed as % of the mean values in normal female mice. Data are the mean ± SEM of eight sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant.

4.5. Expression of proto-oncogenes involved in cell cycle progression

In young adult, 9 weeks old, GH-transgenic mice the proto-oncogenes c-myc, c-jun and c-fos protein content is increased compared to normal controls (Figure 16A-C). Even though particularly c-myc reveals higher protein content for males in normal controls and transgenic animals, no significant sex differences can be detected (Figure 16C). Old animals pursue the findings of young adult mice. All three proto-oncogenes are clearly elevated comparing transgenic to normal siblings. No significant difference can be detected between tumoral and non-tumoral tissue (Figure 17A-C).

Cyclin E and cyclin D1 in 9 weeks old GH-transgenic mice reveal significant elevation of their protein expression compared to normal controls (Figure 16D&E). No sex differences can be detected for cyclin E (Figure 16D). Neither for cyclin D1 a significant sex differences can be observed in the western blot method, except for a tendency to elevation in transgenic males. This tendency to elevation can be confirmed with immunohistochemistry. A statistically significant increase for positive nuclei in transgenic males for cyclin D1 is observed in comparison to female GH-transgenic mice (Figure 16G). The protein content for these two players in old transgenic mice increases significantly in contrast to normal controls (Figure 17D-G). The elevation in cyclin E protein content for tumoral compared to non-tumoral tissue does not differ significantly (Figure 17D). For cyclin D1 no difference between tumoral and non-tumoral zones can be detected via immunohistochemistry. Western blot indicates the same tendency of elevation for cyclin D1 and no difference for tumoral and non-tumoral areas, yet the increase is more noticeable (Figure 17E-G).

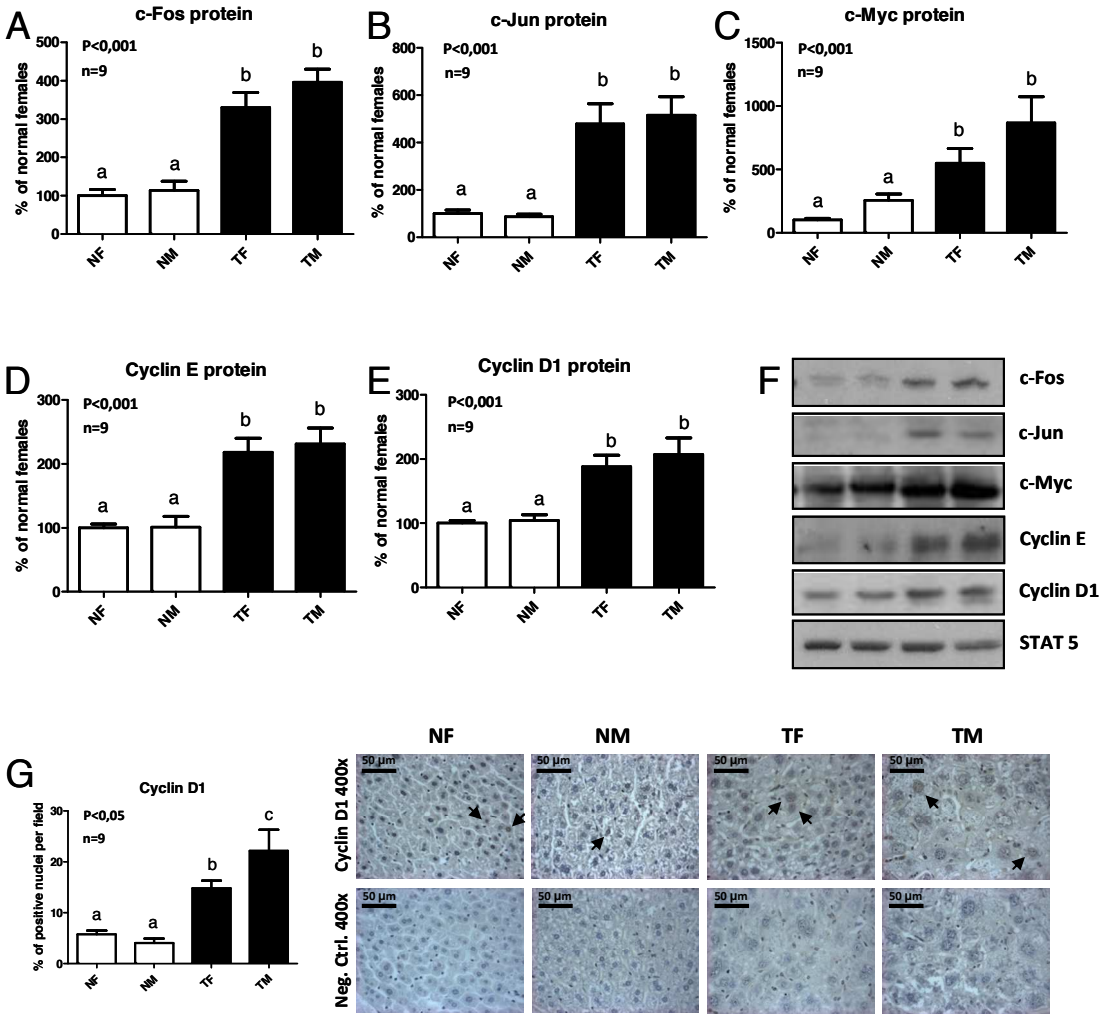

Figure 16. Expression of proto-oncogenes in the liver of young adult GH-transgenic mice and normal controls. Liver extracts from young adult normal female (NF), normal male (NM), GH-transgenic female (TF) and GH-transgenic male (TM) mice were analyzed by immunoblotting to determine protein content. Results are expressed as % of the mean values in normal female mice. (A) c-Fos protein content. (B) c-Jun protein content. (C) c-Myc protein content. (D) Cyclin E protein content. (E) Cyclin D1 protein content. (F) Western blot images of c-Fos protein, c-Jun protein, c-Myc protein, Cyclin E protein, Cyclin D1 protein and STAT5 as loading control. (G) Immunohistochemical staining of liver sections with anti-Cyclin D1 antibody: representative microphotographs are shown; the % of positive hepatocyte nuclei was determined in 10 high-power fields per mouse. Black arrows show representative positive nuclei. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody. Data are the mean ± SEM of nine sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant.

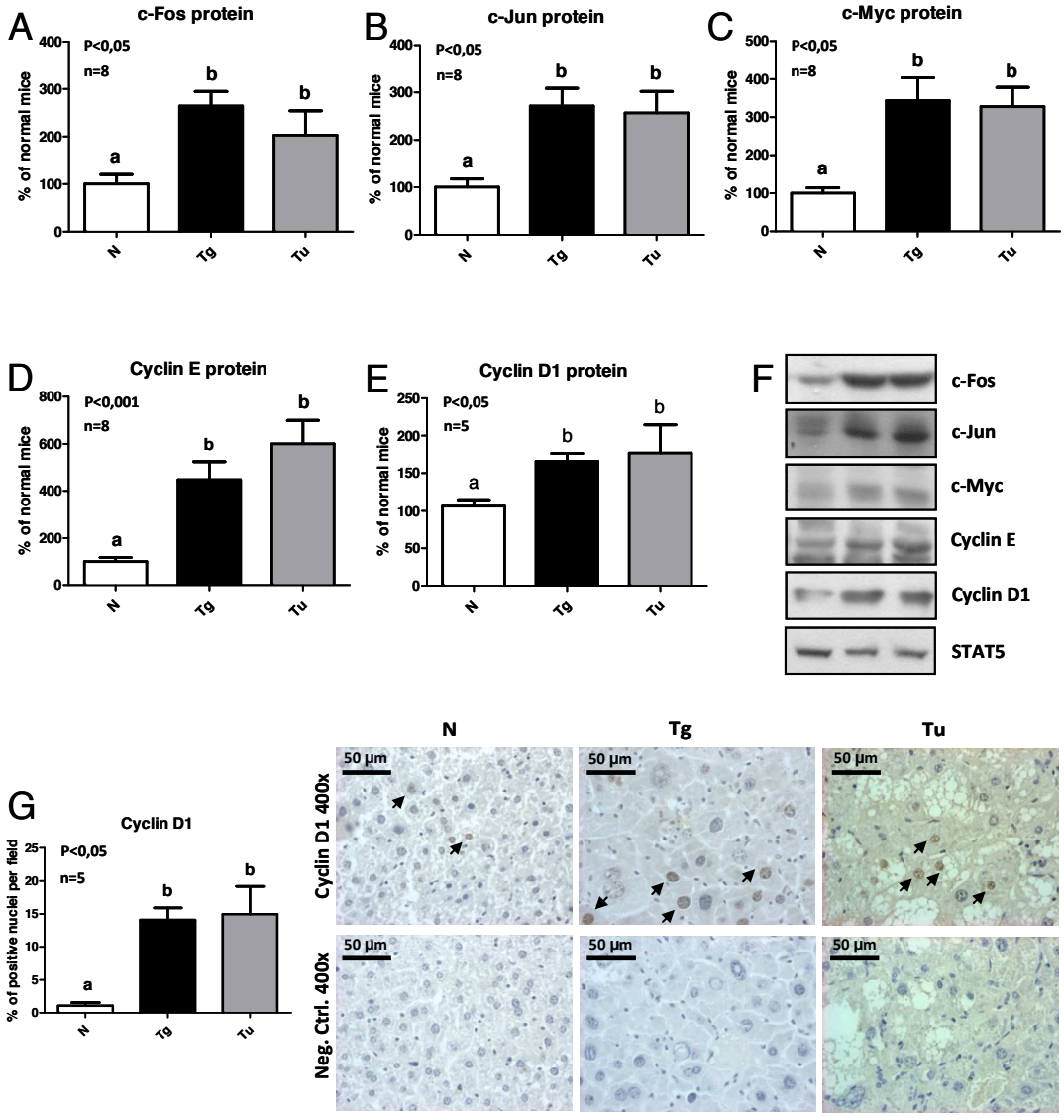

Figure 17. Expression of proto-oncogenes in the liver of old female, more than 1 year of age, GH-transgenic mice and normal controls. Liver extracts from old normal (N) mice, GH-transgenic mice non-tumoral tissue (Tg) and tumoral tissue (Tu) were analyzed by immunoblotting to determine protein content. Results are expressed as % of the mean values in normal female mice. (A) c-Fos protein content. (B) c-Jun protein content. (C) c-Myc protein content. (D) Cyclin E protein content. (E) Cyclin D1 protein content. (F) Western blot images of c-Fos protein, c-Jun protein, c-Myc protein, Cyclin E protein, Cyclin D1 protein and STAT5 as loading control. (G) Immunohistochemical staining of liver sections with anti-Cyclin D1 antibody: representative microphotographs are shown; the % of positive hepatocyte nuclei was determined in 10 high-power fields per mouse. Black arrows show representative positive nuclei. Negative controls were performed in parallel on additional slides and incubated with PBS instead of primary antibody. Data are the mean ± SEM of eight (five for Cyclin D1) sets of different individuals per group (n). Different letters denote significant difference at $p < 0.05$. NS, not significant.

5. Discussion

The aim of this work is to assess the molecular pathogenesis and signal transduction pathways underlying the pro-oncogenic liver pathology induced by prolonged exposure to elevated GH level in mice. Signaling pathways known to participate in the hepatotumorogenesis process were assessed in young adult, 9 weeks old, mice. In addition, it was of interest to evaluate if the progress of the hepatic pathology that is observed with age is accompanied by further changes in the signaling mediators in study. It has been previously reported that female transgenic mice overexpressing GH exhibit hepatic constitutive activation and/or upregulation of several signaling mediators like STAT3, Akt, mTOR, Erk1/2, EGFR and Src (Miquet et al., 2008). This work analyses the expression and activation of downstream components and effectors of these and other signaling pathways, including NF-κB, GSK3-β, β-catenin, c-Myc, c-Jun, c-Fos, cyclin D1 and cyclin E. The sexual dimorphic pulsatile GH secretion and the higher incidence of HCC in males, in humans and in mice (Rogers et al., 2007; Ruggiere et al., 2010) head to analyze whether the molecular alterations described present sexual dimorphism. In GH-transgenic mice no sex differences were reported in terms of tumor incidence and progression (Snibson et al., 1999; Snibson et al., 2001).

Gender differences regard a variety of actions like gene expression, mitochondrial function, metabolic enzymes, microsomal enzyme activity, membrane lipid configuration, immune system and cancer incidence (Rogers et al., 2007; Ruggieri et al., 2010; Baik et al., 2011). In a big range, these differences are supposed to be a consequence of the gender disparity of the GH secretion from the pituitary gland. In both animal models like rats and mice and in humans, males exhibit pulsatile GH release characterized by peaks of high GH concentration and low hormone levels inbetween the peaks in contrast to females, which show more frequent, consistent pulses (Jansson et al., 1985; Jaffe et al., 1998; Tannenbaum et al., 2001; Waxman and O´Connor, 2006). A lot of genes in rapid response to GH signaling are announced to be direct targets of STAT5, including several transcription factors which show sex dependent expression (Wauthier et al., 2008; Wauthier et al., 2010). In direct response to GH signaling STAT5 activity in males is more dynamic and corresponds to the pulsatile manner, while it remains in near continuous lower levels in females

(Tannenbaum et al., 2001; Waxman and O´Connor, 2006; Zhang et al., 2012). The GH pattern-dependent activation of liver STAT5 might be a relevant mediator of GH transcriptional effects in sex-specific liver genes.

In the context of sexual dimorphism this work displays higher hepatocyte cellular and nuclear size in young adult, normal male mice, but cell proliferation was not increased. Beyond this, molecular signaling mediators like STAT5, Akt and GSK3-β showed higher phosphorylation content for young adult-male mice in the normal group. Increased mean STAT5 phosphorylation levels in male mice compared to normal female controls is in line with previous descriptions (Martinez et al., 2013). Pulsatile GH stimulus, as observed in males, leads to stronger activation of STAT5, nuclear translocation and transcriptional activation than the more continuous pattern observed in females, presumably because continuous GH pattern desensitizes hepatic STAT5 signaling. In STAT5 knock out male mice distinguished body growth rates and liver gene expression are declined to wild-type female levels (Udy et al., 1997; Tannenbaum et al., 2001). These findings support the presumption that the increased activation levels of STAT5 are most probably a direct consequence of sex-specific GH secretion patterns.

In this work we have further found that the phosphorylated molecular signaling mediators GSK3-β and Akt were elevated in normal males compared to normal female control. As GSK3-β is a target of the serine/threonine protein kinase Akt, the elevated inhibition of GSK3-β by phosphorylation at Ser9 might be a direct consequence of the higher activation of Akt, reflected by enhanced phosphorylation at Ser473. In accordance to previous observations, no sex differences were detected for other AKT targets like mTOR (Miquet et al., 2010), suggesting different regulation of signaling components downstream Akt. Signal transduction via the PI3K/Akt pathway is known to play a relevant role in hepatocyte cell growth. The exacerbated activation of Akt prescribed in normal males compared to normal females might be associated to the enlarged hepatocellular size in normal males. Recent studies suggest that Akt contributes to liver regeneration mainly by inducing cell size growth rather than cell proliferation (Mullany et al., 2007; Haga et al., 2009). If the observed sex differences in normal mice liver contribute to the higher incidence of HCC in males remains to be elucidated.

Young adult GH-overexpressing transgenic mice from both sexes exhibit hepatomegaly, hepatocyte hypertrophy with enlarged nuclear size and exacerbated hepatocellular proliferation compared to their normal controls, in accordance to previous publication (Orian et al., 1989; Orian et al., 1990; Quaife et al., 1989; Snibson et al., 1999; Hoeflich et al., 2001). The named histological alterations sustain over long time periods in this model and can be detected in old GH-transgenic mice as well. Unexpectedly, in concern of sexual dimorphism, is the observed enlarged hepatocyte cell and nuclear size along with higher proliferation rate in young adult transgenic males compared to transgenic females, as previous reports indicated no significant differences between male and female transgenic mice in terms of tumor incidence and progression (Snibson et al., 1999). Based on those observations, both sexes of GH-transgenic mice were combined in following studies (Snibson et al., 2001; Snibson, 2002), so that possible sexual differences between GH-transgenic male and female mice may have been undetected. Beyond that, proliferation in old transgenic mice is not observed to be diverse between the tumoral and non-tumoral tissue, but it was reported in previous study to rise precisely in tumoral areas (Snibson et al., 1999). The expression and activation of several of the signaling mediators evaluated was exacerbated in transgenic mice compared to normal controls, but no sex differences were detected within the transgenic group, except for nuclear localization of cyclin D1 in males and as observed in further study for higher mRNA levels for cyclin D1 (Miquet et al., 2013).

Young adult GH-transgenic mice in both sexes present a clear tendency to higher phosphorylation levels of STAT5 compared to normal females. Analyzed by ANOVA no statistical significant difference can be found, but it results significant when performed by Student´s t test for female mice only. These findings are in contrast to previous studies using 3–6 months old females, where STAT5 phosphorylation levels were similar in normal and transgenic animals (Miquet et al., 2004; Miquet et al., 2008). That does not apply for males, which exhibit slightly higher STAT5 phosphorylation levels in normal than in transgenic mice. These results seem to contradict a recent publication, in which loss of STAT5 was proposed to controverse alterations induced by persistently high GH levels like increased hepatocyte size and turnover (Friedbichler et al., 2012). It is possible that tyrosine phosphorylation levels of

this protein in whole tissue homogenates are not a direct reflection of the effective activity of STAT5 in GH-transgenic mice, as other factors are involved in its transcriptional activation. Findings in old transgenic animals concerning STAT5 protein content and activated protein in both tumoral and non-tumoral tissue compared to normal controls do not show any significant difference, reflecting previous investigation from our group (Miquet et al., 2004; Miquet et al., 2008).

The serine/threonine protein kinase Akt represents a key figure in cellular processes like growth, proliferation, survival, metabolism, migration and angiogenesis. Increased activation of the Akt pathway due to failure of control mechanisms is associated with a range of malignancies including HCC (Nicholson and Anderson, 2002; Song et al., 2005; Llovet and Bruix, 2008). All three isoforms (Akt1, Akt2, Akt3) contain two regulatory sites, Thr308 and Ser473 (Song et al., 2005). In this work Akt2 was assessed because of its relevance in insulin signaling and growth (Cho et al., 2001; Yang et al., 2004) and it was found to be elevated in the liver of GH-transgenic mice independent from age. Further research reveals no differences in its mRNA levels in young adult transgenic mice (Miquet et al., 2013). A parallel increment in the phosphorylation of the activating residue Ser473, which implicates full activation of the protein, can be observed for young adult and old animals. The slight decrease of Akt2 protein content and its phosphorylation content for tumoral tissue compared with non-tumoral tissue may be related to the correlating decrease in hepatocellular size in tumoral tissue, as Akt is proposed to mainly induce cell size growth (Mullany et al., 2007; Haga et al., 2009). These findings might be relevant, as Akt2 expression is reported to be increased in HCC and to correlate with the progression of the disease, serving as a prognostic factor (Xu et al., 2004). Its dysregulation may constitute a central step in the pro-oncogenic process in GH-transgenic mice liver. Furthermore, failure of Akt regulation is reported to be a critical modulator of increased resistance to chemo- or radiotherapy (Romano 2013; Saini et al., 2013). Akt elevation is actually followed by alteration of downstream components or signaling pathways interacting with Akt, like mTOR and GSK3-β in GH-overexpressing transgenic mice.

The two isoforms known, α and β, of the Ser/Thr Kinase GSK3 are involved in proliferation, differentiation, survival and migration. The β-isoform is analyzed in this work because of its more established role in cell survival (Jope et al., 2007; Jacobs et

al., 2012). GSK3-β is associated with the PI3K/Akt and the Wnt/β-catenin pathways, which are both frequently dysregulated in liver cancer (Jope et al., 2007; Llovet and Bruix, 2008). In addition, β-catenin is involved in a variety of tumorogenesis due to its role in cell-cell adhesion and in cell-cell signaling in development, differentiation and inflammation processes. Quite a high percentage of HCCs show high levels of β-catenin, usually located in the cytoplasm or nucleus rather than at the plasma membrane (Gotoh et al., 2003; Monga, 2011). β-catenin cytoplasmic content is usually kept low by phosphorylation mediated by GSK3, which is part of an inactivating complex that targets β-catenin to proteasome degradation. When GSK3 is inhibited, β-catenin accumulates in the cytoplasm, translocates to the nucleus and acts as a coactivator of transcription factors (Takahashi-Yanaga and Sasaguri, 2008; Jope and Johnson 2004). In GH-transgenic mice elevated levels of GSK3-β phosphorylated at its inactivating residue Ser9 are observed in combination with increased β-catenin content. Given that in further research no increase in β-catenin mRNA level is detected in young adult transgenic mice (Miquet et al., 2013), it can be reasoned that the upregulation of β-catenin is very probably due to increased protein stability rather than higher gene expression. Elevated levels of inactive GSK3-β were reported in HCC harboring β-catenin accumulation (Ban et al., 2003; Monga, 2011). The slight decrease of β-catenin content detected in tumoral tissue compared to non-tumoral tissue might be a consequence of the lower level of relative inhibition of its regulator GSK3-β in these areas. The exacerbation of GSK3-β and β-catenin is in accordance with the elevated expression of downstream oncogenic targets and/or target genes such as cyclin D1, c-Myc, c-Jun and NF-κB. However, the expression of these genes is modulated by additional signaling pathways.

The early delayed response gene in the cell cycle, Cyclin D1, is one of the responsible factors for G1/S transition and in hepatocytes additionally for mediating growth and proliferation (Nelsen et al., 2001; Nelsen et al., 2003). A lot of oncogenes are involved within the signal transduction leading to cyclin expression and cell division. Cyclin D1 is associated with carcinogenesis in many entities and frequently overexpressed in HCC (Joo et al., 2001; Gotoh et al., 2003; Takahashi-Yanaga and Sasaguri, 2008). Some of the signaling mediators in study are involved in cyclin D1 modulation. Amongst others, Akt regulates cyclin D1 levels by multiple and complex mechanisms. Apart

from its interaction on the degradation complex of β-catenin, GSK3-β stimulates cyclin D1 proteolysis through direct phosphorylation and is expected to drive reduction in the cyclin D1 mRNA level as well (Nicholson and Anderson, 2002; Takahashi-Yanaga and Sasaguri, 2008). Therefore, Akt mediated phosphorylation of GSK3-β at Ser9, which inactivates it, would result in the accumulation of β-catenin and cyclin D1. In addition cyclin D1 transcription is induced by NF-κB (Ahn and Aggarwal, 2005) and its mRNA translation is known to be directly influenced through phosphorylation of controling substrates by mTOR (Nicholson and Anderson, 2002). Therefore, the elevated cyclin D1 levels in GH-transgenic mice liver are probably the result of the multiple alterations detected in different signaling pathways downstream Akt. Increase of Cyclin E levels in GH-transgenic mice liver can also be observed over the long term period. Elevation of cyclin E level increases its own transcription via activation of the transcription factor E2F and leads to less inhibition of cyclin D1 through phosphorylation and subsequent degradation of its inhibitor p27 (Duronio et al., 1995).

C-myc, c-fos and c-jun are part of the early genes in the regulation of the priming phase of the cell cycle. Alterations of these protooncogenes appear in many tumors including HCC (Yuen et al., 2001). Apart from the regulation of proliferation, growth and apoptosis, c-myc is a key player in embryonic development (Davis et al., 1993) and is suggested to promote the activity of cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes (Amati et al., 1998). Based on the hypothesis of dependence on epigenetic parameters, the capability of c-myc to induce hepatocarcinogenesis in mice depends on the age of the host. While c-myc overexpression in neonatal mice induced marked cell proliferation and immediate onset of neoplasia, in adult mice it produced nuclear and cellular growth rather than cell proliferation, with malignant transformation only after a prolonged latency period (Beer et al., 2004). The transcription factor complex AP-1, build out of c-Jun homodimer or c-Jun-c-Fos heterodimer, serves the regulation of proliferation, differentiation and cell survival. C-fos expression is reported to be in favour of HCC tissue compared to non-tumoral tissue and its overexpression correlates with high levels of inactive phosphorylated GSK3-β and consequential stabilized cyclin D1 within the nucleus (Yuen et al., 2001; Güller et al., 2008). A former study recognized c-jun as a major regulator of hepatocyte proliferation and survival during liver development and regeneration (Behrens et al., 2002). A Clinical study in patients

with HCC showed high levels of c-Jun in both tumoral and non-tumoral tissue. High levels of c-Fos in contrast were only expressed in tumoral tissue (Yuen et al., 2001). In this work long-lasting elevated expression of c-myc, c-jun and c-fos is observed in the liver of transgenic mice overexpressing GH from young adult to old animals, underlining the reported important role of these protooncogenes in the preneoplastic pathology observed in this model. C-myc overexpression is in line with elevated levels of cyclin D1 and cyclin E, which proposes the mutual interference. In accordance to the previously cited clinical trial are the likewise elevated levels of c-Jun in tumoral and non-tumoral tissue of GH-transgenic mice. However, the higher c-fos expression in tumoral tissue compared to non-tumoral tissue could not be mirrored in this animal model of GH-overexpression (Yuen et al., 2001).

The nuclear factor NF-κB is involved in cell proliferation and survival, immunity and inflammation. Its dysregulation and the subsequent constitutive activation of the nuclear factor is reported in many tumors, including HCC (Ahn and Aggarwal, 2005; Luedde et al., 2011; Hoesel and Schmid, 2013). In most cases it is not associated with genetic alterations, neither in NF-κB and its regulating inhibitor Kinase IK, nor in upstream components (Karin et al., 2002), which supports the thesis of a concrete connection between inflammation and cancer, as HCC is one of the malignancies most established in the context of chronic inflammation triggering tumor development. Through TNF induction, NF-κB comprises the capability of cell death induction and opposite hepatocyte proliferation (Wullaert et al., 2007). NF-κB is known to directly induce the expression of cell cycle related genes including cyclin D1 and c-myc and the range of its impact is extended by crosstalk to different transcription factors and kinases in study, like STAT3 and GSK3-β (Hoesel and Schmid, 2013). Furthermore, survival of immune cells under stress conditions was reported under GH induced NF-κB promotion (Jeay et al., 2002). In this study, NF-κB expression is increased in the liver of GH-transgenic mice compared to normal controls. As a recent study showed increased RelA mRNA and p65 positive inflammatory cells in GH-overexpressing mice (Friedbichler et al., 2012), the increase of NF-κB is most probably the result of the hepatic inflammation in this model. Already mentioned, NF-κB expands its range of modulation via crosstalk to different signaling cascades. Through the expression of the pro-inflammatory cytokine IL-6, it accomplishes STAT3 activation. Both NF-κB and

STAT3 are known to play an important role in liver inflammation (He and Karin, 2011). Consistent with reported high hepatic expression of IL-6 in GH-transgenic mice (Friedbichler et al., 2012), elevated levels of activated STAT3 in this work are shown in both sexes in young adult and in old GH-transgenic mice liver. If the high STAT3 phosphorylation levels are a consequence of GH induced NF-κB expression or due to elevated levels of other growth factors and cytokines, as occurs in this animal model, needs to be elucidated.

In the PEPCK-bGH-transgenic animal model used in this work, GH is mainly expressed in liver, kidney and adipose tissue. To verify that the molecular changes observed in the liver of GH-transgenic mice are a consequence of the elevated circulating GH levels and not the result of the local hepatic expression of the transgene, further investigation in a Mt-GHRH (methallothionein- Growth-hormone-releasing-hormone) transgenic mouse model was made (Miquet et al., 2013). This different line of mice with chronic GH excess shows enhanced production of the endogenous pituitary GH. The phosphorylation and/or protein expression of signaling molecules that were found to be altered in the PEPCK-bGH transgenic line were determined in young female GHRH-transgenic mice. GHRH-transgenic mice display elevated protein abundance and parallel increase in their phosphorylation levels of GSK3-β and NF-κB and higher protein content of β-catenin, cyclin D1, cyclin E, c-Myc, c-Jun and c-Fos in comparison to normal controls (Miquet et al., 2013). A previous report indicated upregulation of Akt, mTOR and high phosphorylation levels of STAT3 as well in GHRH-transgenic mice (Miquet et al., 2008).

The study of molecular alterations associated with oncogenic processes does not only allow improved understanding in the background of pathology, but also in discovering new targets which to date lack the action of pharmaceutics.

6. Conclusion

The use of GH has risen in the last years. Apart from its interactions in growth and metabolism, its use has been approved in different clinical conditions which do not necessarily implicate the lack of GH. Furthermore, GH is used to prevent attributes of aging and to gain better athletic condition, without being approved for these cases. In this context, it should be clear that the use of GH is not free from side effects. A serious considerable side effect of the use or the abuse of GH could be the development of tumors. The opposite was observed in a study in patients with Laron syndrome, carrying a mutation in the GHR gene, leading to severe GH and IGF-1 deficiencies. This population displayed an explicit decrease of cancer and type 2 diabetes in comparison to normal controls (Guevara-Aquirre t al., 2011). The excess of GH is associated with the development of liver tumors in mice. Possible mechanisms for GH promoting this type of cancer imply its effect on cellular proliferation and the modulation of signaling components involved in a big range of processes i.e. inflammation.

The objective of this work was to elucidate the molecular pathogenesis and signal transduction pathways underlying the pro-oncogenic liver pathology induced by prolonged exposure to elevated GH level. The sexual dimorphic pulsatile GH secretion and the higher incidence of HCC in males, both in humans and in mice (Rogers et al., 2007; Ruggiere et al., 2010), bundled the focus in this work on sexual dimorphism within molecular alterations in preneoplastic lesions in young adult GH-transgenic mice compared to their normal controls. The fact that old GH-transgenic mice, more than 1 year of age, frequently develop liver tumors, set the second focus in this work on the deviation in hepatocellular molecular alterations between tumoral and non-tumoral lesions to reach understanding in the ultimate step from preneoplastic to neoplastic lesion. Activation and expression of several downstream components, including NF-κB, GSK3-β, β-catenin, c-Myc, c-Jun, c-Fos, cyclin D1 and cyclin E, of signal transduction pathways that have been implicated in hepatocellular carcinogenesis, like PI3K/Akt and STATs were evaluated in the liver of young adult, 9 weeks old, male and female as well as in old female GH-transgenic mice and their normal controls, to assess its possible association with the liver pathology observed in these animals. Referring to sexual dimorphism in this work, young adult, normal male mice depict higher

hepatocyte cellular and nuclear size, but cell proliferation was not increased. In addition molecular signaling mediators like STAT5, Akt and GSK3-β showed higher phosphorylated activation content for young adult male mice in the normal group. Young adult GH-overexpressing transgenic mice from both sexes exhibit hepatomegaly, hepatocyte hypertrophy with enlarged nuclear size and exacerbated hepatocellular proliferation compared to their normal controls. The expression and activation of several of the signaling mediators evaluated was exacerbated in transgenic mice compared to normal controls, but no sex differences were detected within the transgenic group, except for nuclear localization of cyclin D1 in males. Akt is found to be elevated in the liver of GH-transgenic mice independent from age. Its elevation actually is followed by alteration of downstream components or signaling pathways interacting with Akt, like mTOR and GSK3-β in GH-overexpressing transgenic mice. In GH-transgenic mice elevated levels of GSK3-β phosphorylated at its inactivating residue are observed in combination with increased β-catenin content. Some of the signaling mediators in study are involved in cyclin D1 modulation. Amongst others are Akt, GSK3-β, NF-κB and mTOR. Long-lasting elevated expression of c-myc, c-jun and c-fos is observed in the liver of transgenic mice overexpressing GH from young adult to old animals, underlining the reported important role of these protooncogenes in the preneoplastic pathology observed in this model. The increase of NF-κB and the elevated levels of activated STAT3 in this work are shown in both sexes in young adult and in old female GH-transgenic mice liver. If the high STAT3 phosphorylation levels are a consequence of GH induced NF-κB expression or due to elevated levels of other growth factors and cytokines, as occurs in this mouse model, needs to be elucidated.

Premalignant livers in humans and GH-transgenic mice show some correlations concerning endocrine conditions. Given that GH serum levels are usually increased in cirrhotic patients (Hattori et al., 1992; Kratzsch et al., 1995) and the circadian rhythm of GH secretion gets less and less controlled in these patients (Cuneo et al., 1995), GH-transgenic mice display a very interesting model in premalignant liver disease investigation. Several oncogenic pathways are dysregulated in the liver of both young adult and old GH-transgenic mice, whereby this work marks the molecular aberrations in the process of hepatotumorogenesis. The results of this work give confirmation that prolonged exposure to GH induces liver alterations in signaling pathways which are

involved in cell growth, proliferation and survival, resembling those found in many human tumors. We believe that the long-lasting alterations in these signaling pathways lead to development of liver tumors in old animals. No modification of alteration in the pathways studied is detected consequent to hepatotumorogenesis. The hepatocellular hypertrophy and proliferation found in young adult transgenic mice were exacerbated in males. However, except for higher cyclin D1 in males, the alterations observed for the examined signaling mediators and oncogenes did not present sexual dimorphism in this group. These results suggest that additional molecular mechanisms may account for the higher nuclear and cell size of the hepatocytes in transgenic mice, as well as for the higher proliferation in young adult male transgenic mice. Whether the observed alterations are a direct effect of prolonged GH action on the liver or secondary to other endocrine or metabolic alterations remains to be determined.

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