# Hepatocyte proliferation during liver regeneration is impaired in mice with liver-specific IGF-1R knockout

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**ABSTRACT**

Recent evidence indicates that growth hormone (GH) is involved in liver regeneration. To test whether insulin-like growth factor I (IGF-I) mediates this effect, we studied liver regeneration induced by partial hepatectomy in liver-specific IGF type 1 receptor knockout (LIGFREKO) mice. The absence of IGF-1R caused a significant decrease in hepatocyte proliferation in males (-52%), but not in females, as assessed by Ki67 immunohistochemistry. Cyclin D1 and cyclin A protein levels in the livers of LIGFREKO males were only half those in controls, indicating that cyclin induction during liver regeneration is dependent on IGF-1R signaling. Analyzing the signaling cascade initiated by IGF-1R, we observed a lack of IRS-1 induction in LIGFREKO livers. In contrast, the induction of IRS-2 synthesis was similar in LIGFREKO and control groups, suggesting the existence of differential regulation of IRS synthesis during liver regeneration. Regenerating livers from LIGFREKO animals also showed significantly less activated ERKs than controls. Our findings demonstrate that IGF-1R makes a significant contribution to liver regeneration. Using the LIGFREKO model, we provide new evidence that IGF-1R/IRS-1/ERK signaling may be the intracellular pathway controlling the cell cycle *via* cyclin D1 and cyclin A in the regenerating liver.

*Key words: conditional gene knockout, cyclin, insulin-like growth factor type 1 receptor, insulin receptor substrate, partial hepatectomy.*

## INTRODUCTION

Liver regeneration triggered by partial hepatectomy has been extensively studied in rodent models, to dissect the mechanisms that control hepatocyte proliferation. Following the surgical removal of two-thirds of the liver, hepatocytes exit their mitotically inactive G0 state and progress, largely synchronously, through several cell cycles. This regeneration is controlled by cytokines and growth factors, and generally restores most of the initial liver mass in less than a week in the wild-type (1,2). Clues to the events triggering hepatocyte renewal have been gleaned, in particular, from studies in mutant mice. Liver resection in mice deprived of tumor necrosis factor (TNF) α receptor I (3) or interleukin-6 (IL-6) (4) revealed that TNF-α and IL-6 proteins prime quiescent hepatocytes to exit G0 and enter the G1 mitotic phase. Progression into late G1 and S phase requires growth factors, with hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) acting as prominent hepatic mitogens. Pennisi *et al*. (5) showed that liver regeneration was incomplete and levels of hepatocyte renewal reduced in mice deficient for growth hormone (GH). This suggested a critical role for GH in liver regeneration, but it remained unclear whether GH acted directly on hepatocytes, or indirectly *via* the control of insulin-like growth factor I (IGF-I) production and its cognate receptor IGF-1R, to stimulate liver regeneration.

The tyrosine kinase IGF-1R controls proliferation, differentiation and cell survival in many tissues and cell types. The binding of IGF-I to IGF-1R activates the receptor through rapid tyrosine autophosphorylation and subsequent activation of a family of substrates including insulin receptor substrate-1 (IRS-1), IRS-2 and Shc. Tyrosine-phosphorylated substrates act as docking molecules, by binding proteins containing Src homology-2 domains such as the p85 regulatory subunit of phosphatidylinositol (PI)-3-kinase and Grb2, which activates the PI-3-kinase/Akt and Ras/MEK/ERK pathways (for review, see references 6,7).

IGF-1R protein is expressed strongly in the developing liver, but much more weakly in adults, being virtually undetectable in adult hepatocytes (8-10). In contrast, the ligand is produced in abundance in mature hepatocytes, which are considered to be the main source of circulating IGF-I in adults (11,12).

No role has yet been identified for IGF-1R in adult liver function, and no data suggested that this receptor might be directly involved in postnatal liver growth. However, indirect evidence obtained in experimental models *in vivo* has suggested that the activation of IGF-1R signaling pathways may contribute to the rapid hepatocyte proliferation typically observed during liver regeneration. Accordingly, the liver-specific inactivation of *Igf1* in mice lacking the acid-labile subunit (ALS) delays hepatic regeneration (5). In addition, high levels of IGF-binding protein-1 (IGFBP-1) have been observed during liver regeneration in mice (13) and IGFBP-1 null mutants show abnormal liver regeneration (14,15), indicating that modifications in the bioavailability of IGF-I may be important for proliferating liver cells. Moreover, some (8,16), but not all (5) studies have reported a transient increase in IGF-1R expression in regenerating rodent livers. High levels of IRS-1 and -2 have also been found in rat hepatocytes during late stages of liver regeneration, suggesting that these IGF-1R substrates may play a role in the regulation of hepatic regeneration (17-19).

To investigate the contribution of IGF-1R in hepatocyte proliferation *in vivo*, we developed a mouse model deprived of *Igf1r* gene in hepatocytes (LIGFREKO) using the Cre-lox system, which we then subjected to partial hepatectomy. Conventional IGF-1R knockout mice die shortly after birth (20). In LIGFREKO mice, the absence of *Igf1r* gene did not affect postnatal development or adult liver morphology. Instead, it significantly reduced levels of hepatocyte proliferation during liver regeneration.

## MATERIALS AND METHODS

### Mice

We created a conditional *Igf1r*$^{flox}$ allele by flanking exon 3 of *Igf1r* with loxP sites. *Igf1r*$^{flox/+}$ mice were backcrossed and maintained in both 129/Sv (129) and C57Bl/6 (B6) genetic backgrounds. The excision of exon 3 inactivates the *Igf1r*$^{flox}$ allele (giving the *Igf1r*$^{-}$ allele), which no longer produces IGF-1R (21,22). We obtained an efficient hepatocyte-specific knockout, using mice producing Cre recombinase under control of the albumin promoter and the α-fetoprotein enhancer (*AlfpCre*) (23). *AlfpCre* is expressed in epithelial cells : hepatocytes and cholangiocytes, but in none of the other liver cell types. *AlfpCre* mice were generated by injecting FVB/N zygotes and then backcrossed into B6 genetic background for more than 10 generations. The N10 B6 *AlfpCre* mice were then crossed with B6 *Igf1r*$^{flox/flox}$ mice. *AlfpCre*$^{+/0}$*;Igf1r*$^{flox/+}$ animals were mated with 129 *Igf1r*$^{flox/flox}$ mice, and *Igf1r*$^{flox/flox}$ mice were identified among the offspring. About half the mice were also hemizygous for *AlfpCre,* and therefore developed hepatocyte-specific *Igf1r* gene inactivation. These conditional mutants were named LIGFREKO mice. *Igf1r*$^{flox/flox}$ littermates that had not inherited the *AlfpCre* transgene served as controls. All were B6/129 F1 hybrids and were therefore of identical genetic background. Experiments were performed in accordance with national guidelines for the care and use of laboratory animals. Animals had free access to standard mouse chow and water, and were housed in controlled-temperature rooms under 12/12 h light/dark cycles.

### Genotyping

DNA from skin biopsy samples was genotyped by PCR as previously described (24). Cre-lox deletion of exon 3 in the liver was detected by triplex PCR with the primers 5'-

CCATGGGTGTTAAATGTAATGGC-3', 5'-ATGAATGCTGGTGAGGGTTGTCTT-3' and 5'-ATCTTGGAGTGGTGGGTCTGTTTC-3', as previously described (25).

**Partial hepatectomy**

Mice (7 to 8 months old) were anesthetized by i.p. injection of xylazine (Rompun, Bayer, Germany, 4.8 mg/kg body weight) and ketamine (72 mg/kg, Imalgene 100, Merial, France) and subjected to midventral laparotomy (see Table 1 for cohort sizes). The left lateral, left median and right median lobes were ligated and excised. Partial hepatectomy was performed by a single investigator (C.R.) not informed of the genotype of the mice. After surgery, the abdominal cavity was closed and animals were maintained under standard conditions. Animals were killed at the time points indicated and the regenerating livers harvested. Some of the liver tissue was frozen in liquid nitrogen and stored at -80°C for the extraction of protein or genomic DNA; some was fixed in formalin and embedded in paraffin for microscopy and Ki67 immunohistochemistry, to determine the fraction of hepatocytes that were proliferating. Surgically removed liver lobes and regenerating livers were weighed on a fine balance, and mean resection percentage with respect to mean adult liver size was determined. Mean adult liver size was measured in an independent experiment, using a large number of adult 129/B6 F1 hybrid control mice (male livers 1581 ± 48 mg, female livers 1194 ± 34). Mean liver resection percentage in this series of experiments was 58 ± 2%.

**Histology and immunohistochemistry**

Liver tissue sections (4 μm) were stained with hematoxylin-phloxin-safranin. Hepatocyte proliferation was assessed by Ki67 immunolabeling, using a standardized immunoperoxidase method. Sections were incubated sequentially with an anti-Ki67 antibody (Novocastra Laboratories, Newcastle, UK) at a dilution of 1:100 for 30 min, with peroxidase-conjugated

rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) at a dilution of 1:40 for 40 min and with peroxidase-conjugated pig anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) at a dilution of 1:20. Peroxidase activity was detected using 3-amino-9-ethyl carbazole as the substrate and Mayer's hematoxylin for counterstaining. A microscope eyepiece with a net micrometer (Carl Zeiss, Jena, Germany) at high magnification (x400) was used to count Ki67-positive (Ki67+) hepatocyte nuclei in 10 successive fields from each individual (total surface 0.96 $mm^2$). Standard LacZ staining was performed on liver cryosections (-20°C) from *AlfpCre*$^{+/0}$*;Rosa26R*$^{+/0}$ mice (26). Sections were post-fixed by incubation in 4% paraformaldehyde at 4°C for 10 min, rinsed and stained with X-Gal overnight, counterstained with 1% orange G and photographed with an Olympus BX51 microscope.

**Western blotting and immunoprecipitation**

Liver samples, kept at -80°C until extraction, were homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) and centrifuged at 4°C with 10,000 *g* for 10 min. Protein concentrations were determined from supernatants and 40 µg protein per sample were resolved by SDS-PAGE in 7.5 or 12% acrylamide gels and transferred to nitrocellulose (Amersham Biosciences, Saclay, France). For IRS-1 immunoprecipitation, proteins (500 µg) were incubated with anti-IRS-1 antibody (see below) and protein A/G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 18 hours at 4°C. Immune complexes were collected, washed three times with 0.1% Triton buffer and resolved by SDS-PAGE. For western blotting we used the following antibodies (diluted 1:1,000 unless otherwise indicated): anti-phospho-Akt ($Ser^{473}$), anti-Akt (Cell Signaling Technology, Beverly, MA), anti-IRS-1 (C-20), anti-phospho-ERKs (E-4), anti-ERK1 (C-16), anti-cyclin D1 (A-12, 1:500), anti-cyclin A (C-19, 1:500), anti-

phosphotyrosine (PY99, 1:2,500) (Santa Cruz Biotechnology), anti-IRS-2, anti-phospho-Shc ($Tyr^{317}$), anti-phospho-IRS-1 ($Ser^{307}$) (Upstate Biotechnology, Lake Placid, NY ) and anti β-actin (Sigma, 1:1000). Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences) using Kodak BioMax films.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

*Allele-specific expression assay*. Using total RNA from liver and a triplex RT-PCR we co-amplified fragments from the coding region of the mRNAs specific for the wild-type and the inactivated receptor allele. The single forward primer 5'-CGCCTGGAAAACTGCACG annealed with exon 2, the first reverse primer 5'-AGCTGCCCAGGCACTCCG) bound to a sequence in exon 3, and the second reverse primer 5'-GCAGGGGATACAGTACATGTTT spanned the knockout-specific splice junction between exons 2 and 4. RT-PCR products of 518 bp corresponded to the transcript of the knockout allele, and 574 bp PCR products corresponded to wild-type. We used One-Step RT-PCR and a GeneAmp 2400 PCR cycler. Total RNA was extracted from liver tissue homogenates by RNAXEL, and 50 ng were incubated according to the manufacturer's instructions. Cycling parameters were 2 min at 94°C for initial strand separation and 40 cycles of PCR, each consisting of 30 s at 94, 30 s at 59, and 30 s at 72°C.

*Quantitative RT-PCR*. We used an Applied Biosystems 7300 Real Time PCR System and reactives specific for mouse IGF-1R (AB Mm00802831) following instruction of the supplier. As reference gene expression we chose mouse 18S rRNA (AB 4333760F). The coefficient of variation between duplicata in these experiments was below 10%.

**Statistics**

Means are expressed ± standard error of the mean (SEM). Groups were compared using Student's *t*-test, non-parametric Mann-Whitney U test or $\chi^2$ test, as indicated.

## RESULTS

Efficient hepatocyte-specific inactivation of *Igf1r* gene was obtained using the Cre-lox system. Mice carrying a floxed *Igf1r* allele (*Igf1r*$^{flox}$) (21) were mated with *AlfpCre* transgenic mice that express the Cre recombinase in hepatocytes (23). *Igf1r*$^{flox/flox}$;*AlfpCre*$^{+/0}$ mice were obtained in an F1 129/B6 background (Fig. 1A). They were named LIGFREKO mice, for liver-specific *Igf1r* knockout (25). *Igf1r*$^{flox/flox}$ littermates that had not inherited *AlfpCre* were used as controls. Cre-lox inactivation of *Igf1r* was detectable by genomic PCR in LIGFREKO livers from shortly before birth onwards. The proportion of recombined *Igf1r* knockout alleles rapidly increased, reaching maximum levels of 80% (corresponding to the hepatocyte population) at around day 75, and then remaining stable throughout adult life (Fig. 1B). These recombination kinetics were confirmed by data for the *AlfpCre*-induced activation of a *LacZ* reporter transgene (26; Fig. 1C). Recombination was verified by genomic PCR in each LIGFREKO mouse liver (not shown). RT-PCR analysis showed that IGF-1R was expressed in control liver before, and also after partial hepatectomy, and that LIGFREKO hepatocytes, through cell-type specific Cre-lox recombination, were unable to produce intact IGF-1R mRNA (Details in Fig. 1D). All LIGFREKO mice survived into adulthood, and their body weights did not differ significantly from those of control littermates (Fig. 2A). We did not observe differences in liver mass or liver anatomy between LIGFREKO and control mice. Histological analysis of liver tissue from 8-month-old LIGFREKO and control mice showed no differences in tissue architecture (Fig. 2B).

### Liver regeneration after partial hepatectomy is altered in LIGFREKO mice.

To address the role of the *Igf1r* gene in hepatocyte proliferation *in vivo*, we performed partial hepatectomy in male and female control and LIGFREKO mice. In total, 67 animals (15 females, 52 males) underwent partial hepatectomy (Table 1); peri-operative mortality was 9%,

post-operative mortality 4%. Liver regeneration was examined 40 hours after surgery (T*40*, N = 40), at the time of peak DNA synthesis in mice (1,28). Ki67 levels were also analyzed in a smaller cohort of males at T*48* (N = 10), and protein production was analyzed in another cohort at T*28* (N = 8). Out of 58, four animals (representing both genotypes) showed post-operative complications (Table 1). These four animals also showed no increase in the number of hepatocytes labeled with Ki67 antibodies after surgery, indicating a complete failure of liver regeneration (data not shown). This excluded them from further study.

All pre-operative (T*0*) livers contained very few Ki67+ hepatocytes (males: LIGFREKO 1.2 ± 0.8 Ki67+ cells per field *versus* controls 2.9 ± 1.0, NS, $P = 0.2$; females: LIGFREKO 1.3 ± 0.5 *versus* controls 7.2 ± 2.9, NS, $P = 0.08$), consistent with hepatocytes being mostly quiescent and only rarely dividing. At T*40*, we observed a substantial increase in Ki67+ hepatocytes in all groups (Fig. 3 shows typical immunostaining for Ki67 at T*40*). The prevalence of Ki67+ hepatocytes markedly increased by two orders of magnitude in males during surgically induced liver regeneration, but at T*40* livers from LIGFREKO males contained 52% fewer Ki67+ hepatocytes than livers from control males (144 ± 33 *versus* 303 ± 49; $P < 0.05$, Mann-Whitney test) (Fig. 4A). However, when we analyzed liver regeneration in males at a later time point (T*48* after partial hepatectomy), the difference between genotypes disappeared. This indicated that *Igf1r* gene is required for normal liver regeneration during peak DNA synthesis and that its absence delayed, but did not prevent liver regeneration. None of the studied mice showed histological evidence of liver necrosis. Finally, we confirmed that the proportion of Cre-recombined $Igf1r^{-}$ alleles prior to hepatectomy (≥ 80%) persists in the regenerating LIGFREKO livers (data not shown). This is evidence that new hepatocytes had emerged directly from the IGF-1R-deficient ($Igf1r^{-/-}$) hepatocytes of the non-resected parenchyma. Together, these findings suggest that IGF-1R is involved in hepatocyte renewal after partial liver resection.

In contrast to males, hepatocyte proliferation in females was very similar in LIGFREKO and controls at T*40* (80 ± 42 *versus* 76 ± 32, NS) (Fig. 4B). The number of Ki67+ hepatocytes was thus much higher in livers from control males when compared to those of control females (+300%; $P < 0.02$). This sex-related difference was attenuated in the LIGFREKO group and did not reach statistical significance (+80% in LIGFREKO males with respect to females; NS).

**Induction of cyclin D1 and cyclin A is impaired in regenerating LIGFREKO livers**

We assessed the abundance of cyclins D1 and A, two reliable markers of experimental liver regeneration, by western blotting. Cyclin D1 is induced during the G1 and S phases. Cyclin D1 levels were very similar in LIGFREKO and control mice at T*0* (Fig. 5A). However, by T*40*, cyclin D1 levels had increased eight-fold in control livers but only five-fold in LIGFREKO males. This deficient induction in LIGFREKO mice resulted in cyclin D1 levels being 46% lower than in control animals ($P < 0.05$). This is consistent with the reduction in hepatocyte proliferative response at T*40* (compare with Fig. 3A). Similarly, levels of the S phase-marker cyclin A were significantly lower in LIGFREKO than in control livers at T*40* (-50%; $P < 0.001$) (Fig. 5B). The impaired regeneration of LIGFREKO livers was associated with deficiencies in the induction of cyclin D1 and cyclin A synthesis, suggesting that these proteins are potential targets of IGF-1R signaling in the regenerating liver.

In female LIGFREKO and control mice, cyclin D1 levels increased by a factor of only three between T*0* to T*40*. This increase is markedly smaller than that for any of the male groups, consistent with the levels of hepatocyte proliferation measured in each of these groups. In addition, there was no significant difference in cyclin D1 levels at T*0* or T*40* between female LIGFREKO and control mice (not shown).

**IRS-1 protein expression and ERK activation are deregulated in regenerating LIGFREKO livers**

Increases in the production of IRS molecules have been reported in rat hepatocytes at advanced stages of liver regeneration (17-19). We therefore monitored IRS-1 and IRS-2 levels to determine whether the levels of these proteins increased over time. In control livers, IRS-1 protein levels increased strongly from T*0* to T*28* (+74%, $P < 0.001$), and decreased to almost normal levels from T*28* to T*40* (Fig. 6A). In marked contrast, IRS-1 levels in LIGFREKO livers were already high at T*0*, showed no further increase at T*28*, and had fallen by T*40* (Fig. 6A). Thus, the peak in IRS-1 induction observed at T*28* in controls was absent in LIGFREKO livers. In contrast, no significant differences in the levels of tyrosine-phosphorylated IRS-1 were observed between quiescent and regenerating livers from both groups of animals at T*28* (Fig. 6B). Serine phosphorylation of IRS-1 tended to decrease at T*28* in both groups, and no significant difference was observed when LIGFREKO and control mice were compared (data not shown). Levels of IRS-2 protein increased significantly in control livers at T*28,* but, in contrast to the situation for IRS-1, LIGFREKO and control livers had very similar IRS-2 overproduction profiles (Fig. 6C). Levels of Shc, another signal transducer participating in the IGF-I/IGF-1R proliferative signaling pathway, did not vary between T*0* and T*28*, and showed no genotype-dependent differences (not shown).

The activation of ERKs by phosphorylation downstream from IGF-1R is an essential step in the promotion of cell proliferation (7). We monitored ERK activation by western blotting, using antibodies specific for total or phosphorylated ERKs. Total ERK levels neither varied over time nor depended on genotype, but phosphorylated ERK levels displayed clear differences between LIGFREKO and controls (Fig. 6D and E). Phosphorylated ERK levels in control livers were similar at T*0* and T*28*, and decreased strongly thereafter ($P < 0.002$). In contrast, phosphorylated ERK levels in LIGFREKO livers had already begun to decrease

between T*0* and T*28* ($P < 0.01$) and continued to decrease thereafter ($P < 0.02$) (Fig. 6E). Consequently, LIGFREKO livers contained significantly less activated ERKs at T*28* than control livers. We confirmed this result by directly determining the active fraction of ERK proteins (*i.e.* the individual ratios of phospho-ERKs *versus* total ERKs). Between T*0* and T*28*, the p-ERKs/total ERKs ratio did not change in control livers whereas it decreased significantly (-63%) in LIGFREKO livers ($P < 0.05$; N = 18; data not shown). No differences in Akt phosphorylation were observed between quiescent and regenerating livers at T*28*, and Akt phosphorylation status did not depend on genotype (Fig. 6F; for technical details see Fig. 6G and H). These results suggest that IRS-1 and ERKs are involved in IGF-1R-dependent liver regeneration in males.

Finally, we studied whether the decrease in hepatocyte renewal in LIGFREKO males was correlated with the outcome of surgery or post-operative recovery. An analysis of variables relating to hepatic surgery, including the amount of resected liver tissue and the weight of the regenerating livers (at T*40*), revealed no significant differences between genotypes. However, LIGFREKO males, but not females, lost significantly more body weight after surgery than did controls (Fig. 7A), and this result showed some correlation with the weight of the regenerating livers in males (Fig. 7B). Thus, elevated post-operative weight loss in LIGFREKO males supports the notion that these animals suffer more from partial hepatectomy than control males. Inversely, early post-operative recovery in males may be linked to capacity for hepatocyte renewal, which itself depends on availability of IGF-1R.

## DISCUSSION

LIGFREKO mice had morphologically normal livers and were healthy indicating that, under physiological conditions, hepatocyte IGF-1R is not essential for the maintenance of a normal

adult phenotype. Completion of gene inactivation in hepatocytes using *AlfpCre* was only reached after puberty (see Fig. 1B). Cre-lox recombination occured later than what has been observed with the same *AlfpCre* line but other loxP DNA targets, emphasizing the need to control the timing for every combination of Cre and loxP containing transgenic lines. The rather late recombination observed in LIGFREKO mice precluded investigation of whether hepatic IGF signaling affects early postnatal liver development and, secondarily, general growth. The advantage of *AlfpCre* for the present study is that it efficiently excised *Igf1r*, and that this excision occurred sufficiently late not to engender any significant developmental phenotype. However, recombination of *Igf1r* gene in adult hepatocyte precursor cells can not be excluded and could influence progenitor biology, including their expansion after partial hepatectomy.

*Igf1r* inactivation in LIGFREKO livers markedly altered hepatocyte proliferation after partial hepatectomy at T*40*, but not at T*48*. This is compatible with the notion that the disruption of *Igf1r* gene delayed the progression from G1 to S phase and that the decrease in hepatocyte replication was transient. Mice lacking the ligand IGF-I specifically in the liver also display reduced hepatocyte proliferation during regeneration (5). Those mice also lack ALS, and the relative contributions of ALS and hepatic IGF-I to hepatocyte renewal could therefore not be distinguished. Based on those and our present findings we may now conclude that IGF-I binding to IGF-1R on hepatocytes is important for liver regeneration. Quiescent hepatocytes express little IGF-1R, but their very high levels of IGF-I production, together with increases in IGF-1R expression during liver regeneration, seem to be sufficient for significant biological IGF action. The activation of IGF-1R-dependent signaling pathways may also enhance the action of other mitogens during the G1 to S transition. Consistent with this, IGF-I has been identified as a co-mitogen for HGF in murine hepatocellular carcinomas (30), and EGF induction of ERKs in rat hepatocytes has been shown to require IGF-1R

transactivation by EGFR (31). We hypothesize that lack of IGF-1R conditions the liver in a way that it cannot fully respond to the stimulus triggered by partial hepatectomy,

We found a defective liver regeneration in LIGFREKO males, but not in females. Moreover, control females had considerably fewer Ki67+ hepatocytes than males. Thus, the sex-related difference in hepatocyte regeneration disappeared in mice lacking hepatic IGF-1R, suggesting that sex-dimorphism in liver regeneration may involve signal transduction *via* IGF-1R. Similar IGF-1R-dependent dimorphisms have been reported for ageing and resistance to oxidative stress (22,27,32). Liver regeneration in rodents has been reported to depend on sex (33,34), with females having a delayed DNA synthesis peak (35,36). It therefore remains possible that IGF-1R plays a role in liver regeneration in females, but at a time-point later than T*40*. It is also possible that animal age (7-8 months in these experiments) plays a more salient role in females than in males such that in females the effect of IGF-1R on liver regeneration was abolished.

Studies in liver cells derived from mice with knockouts for insulin receptor and IRS have shown that IRS-1 is the main effector of proliferative signals from IGF-1R, whereas IRS-2 is essential for mediating responses to insulin (37). In addition, transgene-mediated overproduction of IRS-1 in the liver increases the number of hepatocytes (38). We observed changes in the abundance pattern of IRS-1, but not in that of IRS-2, in livers from LIGFREKO males in the transition from quiescent to regenerative states. This differential regulation suggests that the production of IRS-1, but not that of IRS-2, is controlled by IGF-1R-dependent pathways. These data support the idea that IRS-1 is a major substrate of IGF-1R in the liver.

Several lines of evidence suggest that IRS-1 protein levels are tightly controlled by insulin and IGF-I *in vitro*. Long-term exposure to either ligand promotes IRS-1 ubiquitination

and degradation by the proteasome (39-41). Our findings may therefore indicate defective IRS-1 turnover in the absence of IGF-1R, leading to IRS-1 accumulation in quiescent hepatocytes. As the upregulation of IRS-1 is strongly associated with liver regeneration (17-19; this study), the abundance of IRS-1 in quiescent LIGFREKO livers may prevent the subsequent induction of IRS-1 production, thereby affecting IRS-1-dependent proliferative responses.

Concomitantly with the reduction of hepatocyte proliferation in LIGFREKO males, we observed a significant decrease in cyclin D1 and A induction. IGF-I has been reported to stimulate the proliferation of other types of cell by promoting G1 to S phase progression and cyclin D1 induction (42-45). Our data indicate that cyclin D1 may be a target of IGF-1R-dependent pathways in the liver. *In vitro*, IGF-I increases cyclin D1 production by stimulating ERK1/2- and PI-3K/Akt-dependent mechanisms (43,44). Although we observed no modulation of Akt phosphorylation at T*28*, the marked decrease in ERK phosphorylation in LIGFREKO livers may be involved in decreasing hepatocyte proliferation.

In light of the results presented here, suggesting that IGF-1R plays a significant role in hepatocyte regeneration, we re-examined the LIGFREKO phenotype at T*0*, searching for subtle changes indicative of some pre-existing function for IGF-1R in normal liver: if pre-operative Ki67+ values from male and female LIGFREKO mice were combined, then they were significantly lower than those of controls (1.2 ± 0.6 *versus* 3.7 ± 1.0, $P = 0.05$). Together with the conspicuously lower cyclin A levels at T*0* (see Fig. 5B) and the tendency for IRS-1 levels to be high at T*0* (Fig. 6A), this raises the possibility that LIGFREKO animals had already developed minor phenotypic differences from controls before surgery. Such conclusion is consistent with the finding that DNA content per unit wet weight of tissue at T*0* was 14% lower in LIGFREKO than in control mice (343 ± 14 vs 401 ± 16 µg/g, $P < 0.02$, n =

29). This may indicate a higher mean hepatocyte volume and/or a higher percentage of tetraploid hepatocytes in mutants, that both merit further investigation.

Comparing the liver regeneration phenotypes of LIGFREKO with IGFBP-1$^{-/-}$ mice (14,15) we found similitudes (delayed regeneration) but also differences (IGFBP-1$^{-/-}$ show significant liver necrosis upon partial hepatectomy). The alterations in intracellular signaling events observed in both models also overlap (cyclin A diminished or delayed) and differ (cyclin D1 close to normal in IGFBP-1$^{-/-}$). Thus it seems possible that part of the effects of IGFBP-1 on liver regeneration depends on signaling via IGF-1R. Similarly, the recent liver-specific knockout of transcription factor STAT-3 (46) has shown that this factor plays a major role in liver regeneration, but also that not all of the IL-6 effects on hepatocyte proliferation are mediated by STAT-3. Together, these studies show convincingly that through targeted genetic approaches the molecular mechanisms of liver regeneration can be progressively elucidated.

In conclusion, the present findings together with previous work (5) indicate that intact GH/IGF-I/IGF-1R signaling pathways are required for normal liver regeneration. In this process, IRS-1, rather than IRS-2, seems to be responsible for transduction of the proliferative response downstream from IGF-1R in the regenerating liver, *via* the activation of ERKs, cyclin D1 and cyclin A.

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**Table 1**

Cohort sizes from liver surgery to analysis

| Group | No. undergoing surgery | Mortality | | Post-operative complication[1] | Final study group | |
|---|---|---|---|---|---|---|
| | | Operation | Post-OP | | N | % of no. undergoing surgery[2] |
| Male LIGFREKO | 22 | 3 | 1 | 0 | 18 | 82% |
| Male Control | 30 | 2 | 1 | 2 | 25 | 83% |
| Female LIGFREKO | 7 | 0 | 1 | 1 | 5 | 71% |
| Female Control | 8 | 1 | 0 | 1 | 6 | 75% |
| All mice | 67 | 6 | 3 | 4 | 54 | 81% |

[1] Animals not freely moving in cage after 24 hours were considered to be suffering from post-operative complications.

[2] Cohort size of the final study group with respect to the total number of mice undergoing surgery.

**FIGURE LEGENDS**

**Figure 1.** Liver-specific *Igf1r* knockout (LIGFREKO). **A**) Breeding strategy for the generation of homozygous *Igf1r*$^{flox/flox}$;*AlfpCre*$^{+/0}$ mice and *Igf1r*$^{flox/flox}$ control littermates in a 129/B6 F1-hybrid genetic background. Triangles indicate loxP sites, rectangles indicate exon 3 of *Igf1r*, red indicates homozygous *Igf1r* knockout in the liver. **B**) Cre-lox recombination in LIGFREKO mice as a function of age, determined by genomic PCR (24) in *Igf1r*$^{flox/wt}$;*AlfpCre*$^{+/0}$ mice (n = 32). **C**) Developmental Cre-lox recombination, assessed using a reporter transgene (26). Blue X-gal staining in liver tissue from *AlfpCre*$^{+/0}$;*Rosa26R*$^{+/0}$ mice indicates Cre-lox-recombinant cells. 50 µm scale bar. **D**) Quantitative RT-PCR demonstrating IGF-1R expression in wild-type liver (left panel). The prevalence of IGF-1R mRNA relative to 18S rRNA increased from T*0* to T*48*. AU, arbitrary units. Triplex RT-PCR (27) showed that wild-type and knockout IGF-1R mRNAs were simultaneously produced in LIGFREKO livers (right panel). Because only LIGFREKO hepatocytes contain knockout alleles (lacking exon 3) this result demonstrates that they express IGF-1R mRNA, before and after partial hepatectomy. Knockout mRNA cannot give rise to receptors. Hence, LIGFREKO hepatocytes (and possibly also their precursors) lost their ability to generate IGF-1R, while Kupffer cells and/or blood vessel endothelia continuously express functional (wild-type) IGF-1R. The triplex RT-PCR amplification from liver RNA was however not quantitative. WM, weight marker for DNA; bp, basepairs.

**Figure 2.** Postnatal phenotype of LIGFREKO mice. **A**) Body growth of LIGFREKO mice (in red) with respect to controls. Differences in weight between genotypes were observed in males at around 8-12 weeks of age, but did not reach significance or persist into adulthood. **B**)

Male LIGFREKO and control liver samples. Hematoxylin-phloxin-safranin (HPS) staining did not reveal changes in liver tissue structure.

**Figure 3.** Liver histology and Ki67 immunohistochemistry in LIGFREKO and control mice at T*40*. **A** and **B**) Hematoxylin-phloxin-safranin (HPS) staining showed no hemorrhage or necrosis at T*40*. **C** and **D**) Representative examples of Ki67 immunohistochemistry in LIGFREKO and control males. **E** and **F**) Representative examples of Ki67 immunohistochemistry in LIGFREKO and control females. Arrows indicate Ki67+ hepatocytes. The limited abundance compared to the literature of Ki67+ hepatocytes at T*40* may be due to more advanced age of the animals (7-8 months in this study), their genetic background and/or the fact that liver resection was slightly below two thirds.

**Figure 4.** Quantitation of hepatocyte proliferation by Ki67 immunohistochemistry. For each of the 36 animals included in the study at T*40*, ten micrometric fields were evaluated. **A**) Male LIGFREKO (n = 8) *versus* male control group (n = 17) at T*0* and at T*40.* **B**) Female LIGFREKO (n = 6) *versus* female control group (n = 5). The density of hepatocytes was similar in LIGFREKO and control livers (males: LIGFREKO 208 ± 16 cells per field vs. control 220 ± 6, -5.5%, $P = 0.42$; females: LIGFREKO 238 ± 8 cells per field vs. control 233 ± 10, +2.1%; $P = 0.72$). Results expressed as Ki67+ counts per hepatocytes were very similar to the displayed Ki67+ expressed per observation field (males: LIGFREKO 14.6 ± 3.9% vs. 26.9 ± 4.4; females: LIGFREKO 8.6 ± 3.6% vs. controls 7.2 ± 2.5). Average liver resection in females was 60%, and thus not lower than in males. Mitotic figures were counted at T*0* and T*40* and were found strongly correlated with the prevalence of Ki67+ cells at T*40* (R = 0.77, $P < 0.0001$, all 4 groups combined, not shown).

**Figure 5.** Cyclin D1 and A expression in male mice before and after partial hepatectomy by western blotting. The bar graphs represent mean ± SEM of scanning densitometry analysis of a total of 13 blots. Quantitative results are expressed relative to the control group at T*40* (100%). **A**) Bar graph: Cyclin D1 expression at T*40* was significant lower in LIGFREKO livers compared to controls. Right panel: Time course of cyclin D1 induction after partial hepatectomy in control mice. Cyclin D1 was slightly induced at T*28* and peaked at T*40* consistent with published data (29). **B**) Bar graph: Cyclin A expression at T*40* was significantly lower in LIGFREKO livers compared to controls. To illustrate the quality of signals, one blot representing a single animal from each group is shown next to each bar graph. Arrows indicate bands of interest.

**Figure 6.** Signal transduction components downstream from IGF-1R before and after partial hepatectomy. The prevalence and/or phosphorylation of IRS-1 (**A** and **B**), IRS-2 (**C**), ERKs (**D** and **E**), and Akt (**F**) were examined by western blotting. Each bar graph represents mean ± SEM of scanning densitometric analysis from, on average, 14 blots. Quantitative results are expressed relative to the control group at T*0* (100%). Total ERKs (**D**) revealed constant over time and levels did not depend on genotype, such that it may serve as control and reference for other proteins. **G**) To illustrate the quality of blotting signals, one blot representing a single animal from each group is depicted. Arrowheads indicate bands of interest. ND, not determined. **H**) β-actin showed strong overexpression in the transition from T*0* to T*40* and thus appeared less suited as western blot loading control.

**Figure 7.** Body and liver weight after surgery: comparison of LIGFREKO and control mice at T*40*. **A**) Body weight was determined before anesthesia and immediately before killing the partially hepatectomized animals at T*40*. **B**) Liver mass after 40 hours of regeneration. The

highest liver weight occurred in the control male group that also suffered the least from postoperative weight loss.

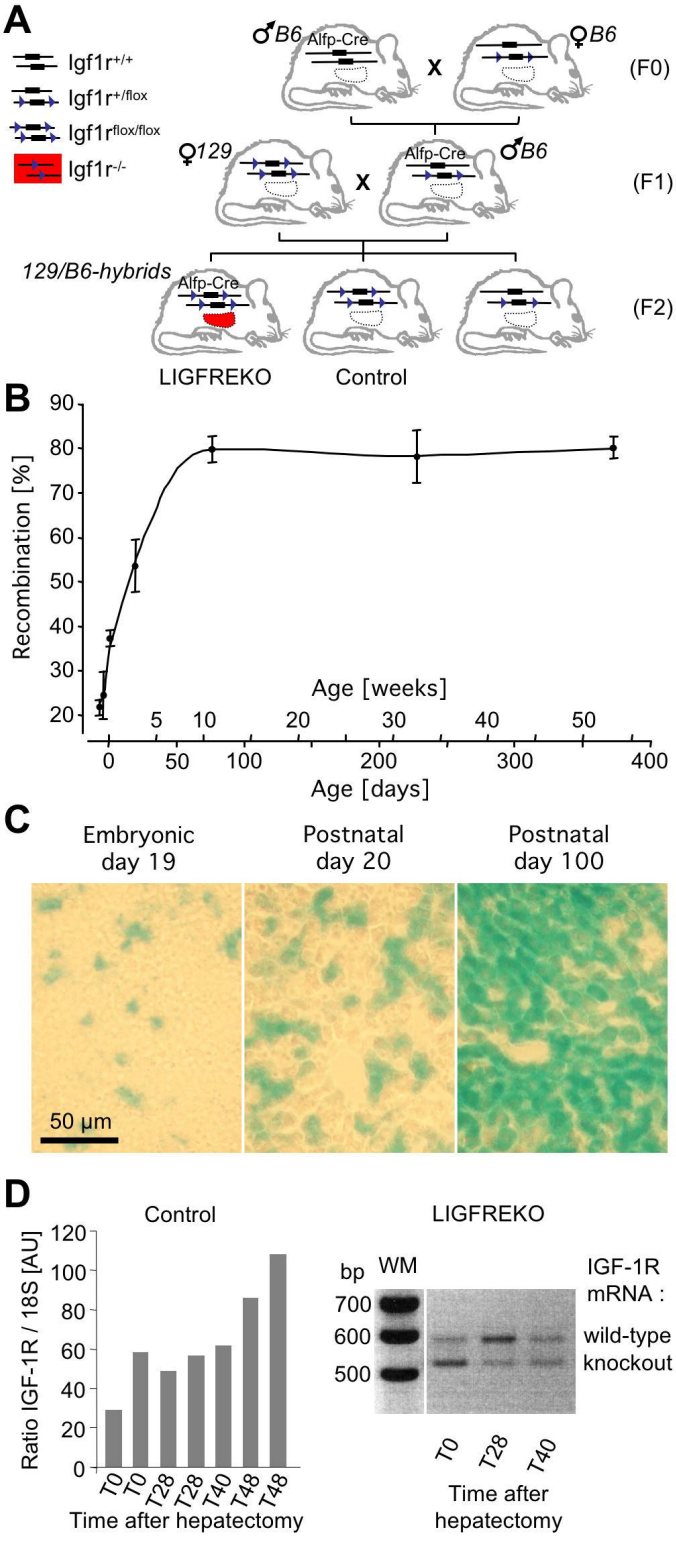
A
Igf1r+/+
Igf1r+/flox
Igf1rflox/flox
Igf1r-/-
♂B6 Alfp-Cre
x
♀B6
(F0)
♀129
x
Alfp-Cre ♂B6
(F1)
129/B6-hybrids
Alfp-Cre
(F2)
LIGFREKO
Control
B
Recombination [%]
90
80
70
60
50
40
30
20
Age [weeks]
5
10
20
30
40
50
0
50
100
200
300
400
Age [days]
C
Embryonic day 19
Postnatal day 20
Postnatal day 100
50 µm
D
Control
LIGFREKO
Ratio IGF-1R / 18S [AU]
120
100
80
60
40
20
0
T0
T0
T28
T28
T40
T48
T48
Time after hepatectomy
bp
WM
700
600
500
IGF-1R mRNA :
wild-type
knockout
T0
T28
T40
Time after hepatectomy

Fig. 1

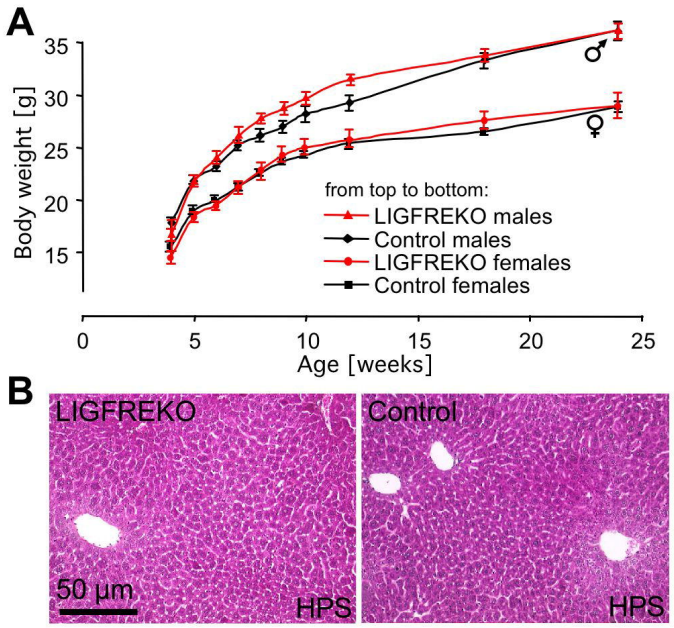
A
Body weight [g]
35
30
25
20
15
0
5
10
15
20
25
Age [weeks]
from top to bottom:
LIGFREKO males
Control males
LIGFREKO females
Control females
♂
♀
B
LIGFREKO
Control
50 µm
HPS
HPS

Fig. 2

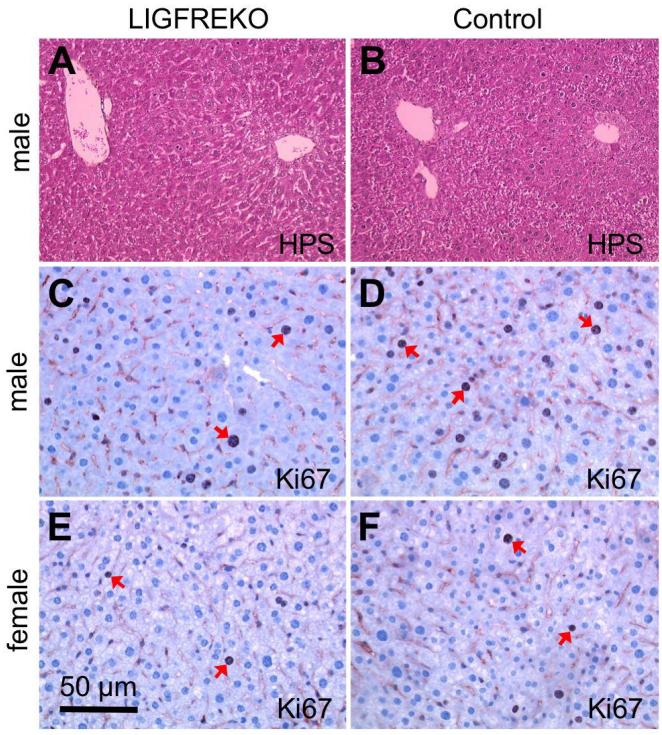
LIGFREKO
Control
A
B
male
HPS
HPS
C
D
male
Ki67
Ki67
E
F
female
50 µm
Ki67
Ki67

Fig. 3

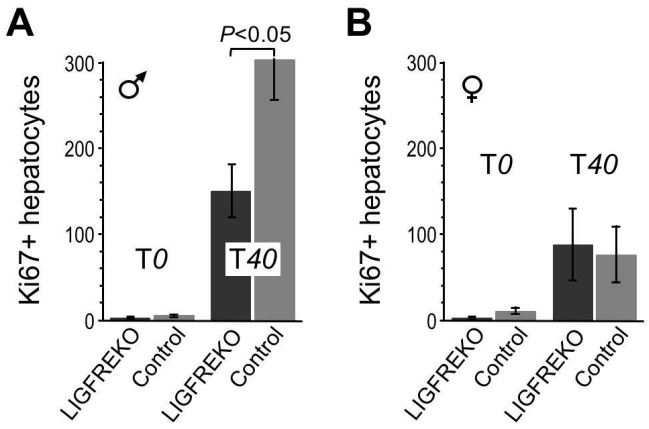
A
B
P<0.05
Ki67+ hepatocytes
300
200
100
0
♂
♀
T0
T40
LIGFREKO
Control
LIGFREKO
Control

Fig. 4

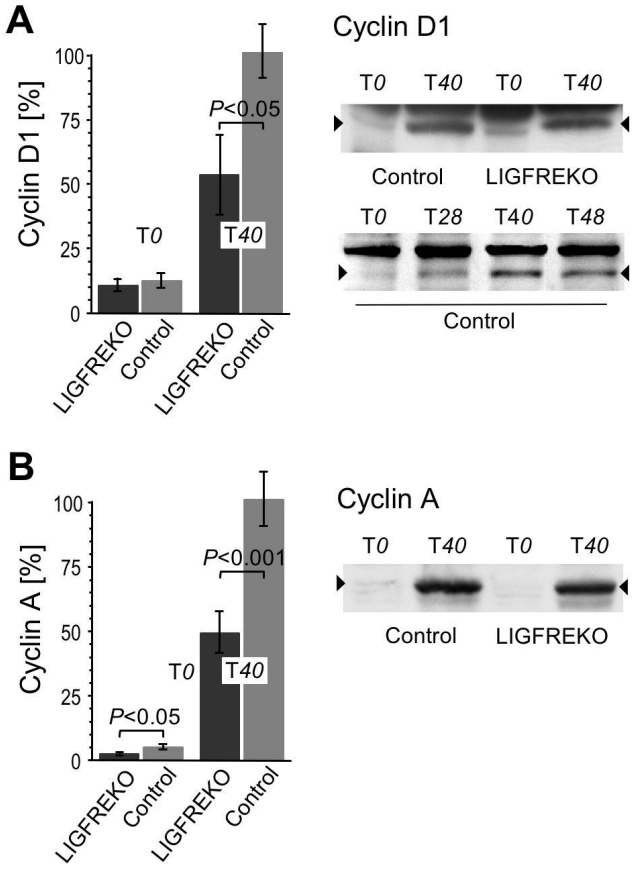
A
Cyclin D1 [%]
100
75
50
25
0
P<0.05
T0
T40
LIGFREKO
Control
LIGFREKO
Control
Cyclin D1
T0
T40
T0
T40
Control
LIGFREKO
T0
T28
T40
T48
Control
B
Cyclin A [%]
100
75
50
25
0
P<0.001
P<0.05
T0
T40
LIGFREKO
Control
LIGFREKO
Control
Cyclin A
T0
T40
T0
T40
Control
LIGFREKO

Fig. 5

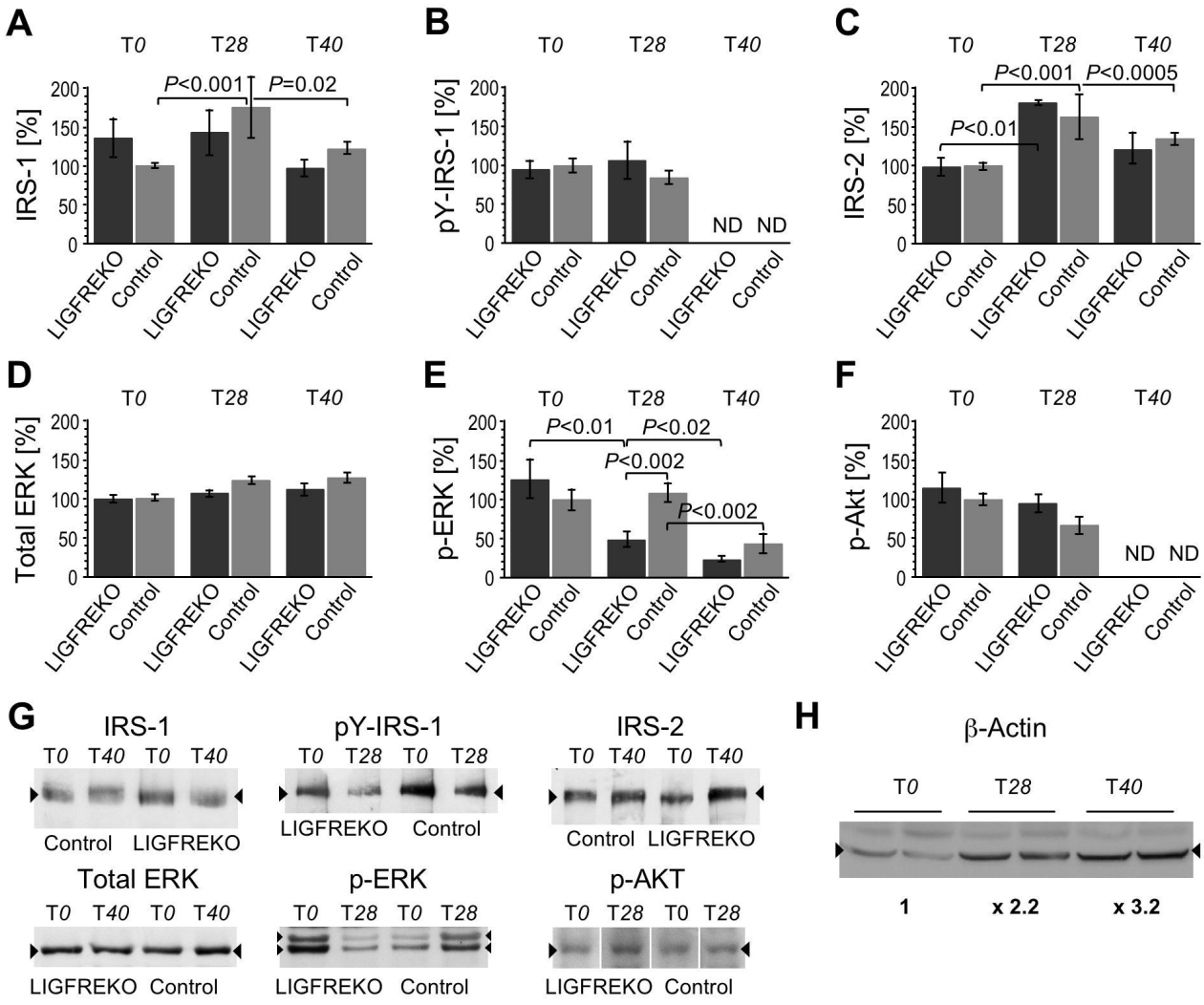
A
T0
T28
T40
P<0.001
P=0.02
IRS-1 [%]
LIGFREKO
Control
B
pY-IRS-1 [%]
ND ND
C
P<0.001
P<0.0005
P<0.01
IRS-2 [%]
D
Total ERK [%]
E
P<0.01
P<0.02
P<0.002
P<0.002
p-ERK [%]
F
p-Akt [%]
G
IRS-1
pY-IRS-1
IRS-2
Total ERK
p-ERK
p-AKT
H
β-Actin
1
x 2.2
x 3.2

Fig. 6

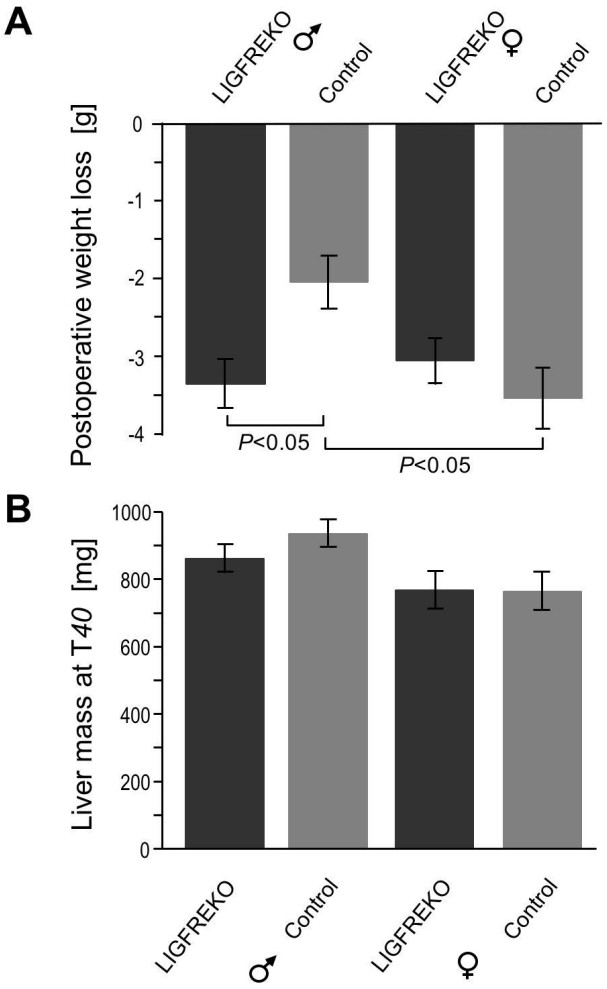
A
LIGFREKO ♂
Control
LIGFREKO ♀
Control
Postoperative weight loss [g]
0
-1
-2
-3
-4
P<0.05
P<0.05
B
Liver mass at T40 [mg]
1000
800
600
400
200
0
LIGFREKO
♂
Control
LIGFREKO
♀
Control

Fig. 7