

Insulin-like growth factor binding protein-3 is required for the regulation of rat oval cell proliferation and differentiation in the 2AAF/PHX model

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Abstract: Oval cell-mediated liver regeneration is a highly complex process that involves the coordination of several signaling factors, chemokines and cytokines to allow for proper maintenance of the liver architecture. When hepatocyte proliferation is inhibited, an hepatic stem cell population, often referred to as “oval cells”, is activated to aid in liver regeneration. The function of insulin-like growth factor binding protein-3 (IGFBP-3) during this process of oval cell activation is of particular interest because it is produced in liver and has been shown to induce migration and differentiation of other stem cell populations both *in vitro* and *in vivo*. Additionally, IGFBP-3 production has been linked to the transforming growth factor- β (TGF- β) superfamily, a pathway known to be induced during oval cell proliferation. In this study, we set out to determine whether IGFBP-3 plays a role in oval cell proliferation, migration and differentiation during this specific type of regeneration. Through activation of the oval cell-mediated liver regeneration in a rat model, we found that IGFBP-3 is elevated in the liver and serum of animals during peak days of oval cell activation and proliferation. Furthermore, *in vitro* assays found that WB-344 cells, a liver stem cell line similar to oval cells, were induced to migrate in the presence of IGFBP-3. When expression of IGFBP-3 was knocked down during oval cell activation *in vivo*, we found that oval cell proliferation was increased and observed the appearance of numerous atypical ductular structures, which were OV-6 and Ki67-positive. Finally, quantitative real-time PCR analysis of liver tissue from IGFBP-3 small interfering RNA (siRNA) treated animals determined that expression of TGF β family members, including TGF- β RII and Smads 2–4, were significantly downregulated compared to animals at day 9 post-PHx alone or animals that received negative control siRNA. In conclusion, IGFBP-3 may function as a potent chemoattractant of oval cells during specific types of liver regeneration and may be involved in regulating oval cell proliferation and differentiation *in vivo* via the TGF- β pathway.

Keywords: hepatic stem cells, transforming growth factor-beta, N-2-acetylaminofluorene (2AAF), partial hepatectomy (PHx)

Introduction

The liver is a unique organ in that it is capable of rapid regeneration in response to chemical damage or physical injury. In addition to this, the liver also contains a stem cell population that, when activated, is responsible for liver regeneration when hepatocyte proliferation is inhibited.¹ Liver regeneration in the rat via these hepatic stem cells, or oval cells, can be identified by the presence of small infiltrating cells,

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approximately 10 μm in size with ovoid nuclei and are located in and around the periportal regions of the liver lobule.² Oval cells can also be identified by their expression of specific markers such as OV-6, α -fetoprotein (AFP), and CK19.²⁻⁴

Activation of the oval cell compartment in the rat model is accomplished through the implantation of an N-2-acetylaminofluorene (2AAF) pellet to prevent proliferation of the resident hepatocytes, followed by a 70% partial hepatectomy (PHx).^{3,5} This model is considered the gold standard in oval cell activation and is commonly referred to as the 2AAF/PHx protocol.³ Oval cells are capable of generating both hepatocytes and bile duct cells, thereby qualifying them as bipotential progenitor cells in adult livers.⁶

Insulin-like growth factor binding protein-3 (IGFBP-3) is one of six characterized binding proteins to insulin-like growth factor-I (IGF-I) and is the most abundant IGF binding protein found in circulation.⁷ IGF-I is an endocrine hormone predominantly synthesized in the liver, and has been shown to play a role in the growth and proliferation of nearly every bodily cell. IGFBP-3 plays an important role in regulating IGF-I function by binding to approximately 75% of free IGF-I in the serum.⁸ By binding to IGF-I, IGFBP-3 has been found to regulate the bioavailability of IGF-I as well as prolong its half-life in circulation.⁹ In addition to regulating the bioactivity of IGF-I, several groups have identified additional functions of IGFBP-3, which do not involve the presence of IGF-I protein. Many of these IGF-I independent functions, including both inhibiting and enhancing cell proliferation, were discovered in cells which either lacked the IGF-I receptor or in cells which are unresponsive to IGF-I.^{10,11}

Recently, IGFBP-3 has been shown to act as a potent inducer of hematopoietic stem cell migration and differentiation both *in vitro* and *in vivo*.¹² Due to the fact that IGFBP-3 is produced in the liver and has been shown to activate other stem cell populations *in vivo*, we asked the question of whether or not IGFBP-3 could potentially play a role in regulating the migration, proliferation and/or differentiation of the hepatic oval cells during oval-cell mediated liver regeneration.

Multiple signaling pathways have been shown to contribute to both early and late events in oval cell activation, which are initiated by several growth factors including α -fibroblast growth factor (α FGF), hepatocyte growth factor (HGF), transforming growth factor (TGF)- β , and TGF- β 1.^{13,14} In addition to this, it has been shown that activation of the

TGF- β pathway induces both IGFBP-3 mRNA and protein expression.¹⁵ IGFBP-3 has also been shown to directly regulate cell proliferation through specific interactions with TGF- β receptors, such as TGF- β RV and TGF- β RII, and TGF- β dependent signaling mechanisms, such as Smads 2, 3 and 4.¹⁶ In addition to this, previous research has also shown a correlation between peak days of oval cell proliferation in the 2AAF/PHx model and increasing levels of TGF- β 1 in the liver.^{1,17} Due to the fact that TGF- β 1 is highly expressed in the liver during oval cell-mediated liver regeneration and shown to be involved in the upregulation of IGFBP-3 protein, we asked whether the effects of IGFBP-3 on oval cells during regeneration are dependent on the activation of the TGF- β pathway and if suppression of IGFBP-3 via siRNA is associated with changes in the expression of genes associated with the TGF- β family.

Materials and methods

AAF implantation and partial hepatectomy

All procedures were performed on male Fisher344 rats (Charles River Laboratories Inc., Wilmington, MA) approximately 8 to 10 weeks old. Upon anesthetization a 28 day time release 2AAF pellet (Innovative Research Inc., Sarasota, FL) was implanted in the peritoneal cavity of the animal. One week later, a 70% surgical resection of the liver was performed under general anesthesia as previously described.¹⁸ For 2AAF/PHx time course experiments, rats ($n = 3$ per time point) were euthanized by injection of sodium pentobarbital (0.1 mL/100 g). Livers and serum were collected at days 0, 3, 5, 7, 9, 11, 13, 15, 17 and 22 post-PHx. For all siRNA experiments, animals again received a 28 day time release 2AAF pellet and underwent 70% partial hepatectomy a week later. Animals were euthanized by injection of sodium pentobarbital (0.1 mL/100 g) and animal serum and livers were collected at day 9 post-PHx only. All procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

Migration assay

Cell motility was assessed in transwells as previously described.¹⁹ WB-344 cells were plated in transwells (1×10^5 cells/well) in Dulbecco's modified Eagle medium F12 (DMEM/F12) 50:50 (10% fetal bovine serum [FBS]) and allowed to attach overnight at 37°C, 5% CO₂. IGFBP-3 (Millipore Corp, Billerica, MA) was added to the lower chamber of the transwell plates at various concentrations

(0.1 ng/mL, 1 ng/mL, and 10 ng/mL). The assay was allowed to continue at 37°C, 5% CO₂ for 6 hours prior to termination. As a negative control, some lower wells contained only migration buffer (without IGFBP-3), IGFBP-3 at 1 ng/mL added to the upper wells only, or IGFBP-3 added to both the lower and upper wells. Migration assays were performed a minimum of three times to ensure statistical significance.

MTT proliferation assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ([MTT], Sigma-Aldrich, St. Louis, MO), assay was performed as previously described.²⁰ WB-344 cells (1×10^4) were seeded in 96 well plates in DMEM/F12 50:50 (10% bovine serum albumin [BSA]) and were incubated at 37°C, 5% CO₂, overnight. The next day, medium was removed and the cells were cultured in DMEM/F12 50:50 with 0.5% BSA containing either IGFBP-3 (1 ng/mL) alone, IGF-I (1 ng/mL) alone, or IGFBP-3 with IGF-I (both 1 ng/mL). Plates were cultured for 24 to 48 h and then analyzed using spectrophotometer at a wavelength of 570 nm.

Histology, immunohistochemistry, and immunofluorescence

For morphology studies, 5 µm paraffin sections were stained with hematoxylin and eosin (H & E). Immunohistochemistry and immunofluorescence (IF) were performed either on 5 µm paraffin embedded or optimum cutting temperature (OCT) frozen sections. The following antibodies were utilized: Ki67 at 1:100 (556003, BD Pharmingen, San Jose, CA), IGFBP-3 at 1:100 (sc-6004, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and OV-6 at 1:150 (a generous gift from Dr Stewart Sell, Ordway Research Institute; Albany, NY). Staining was visualized using the appropriate biotinylated secondary antibody with the Vectastain Elite kit and 3,3'-diaminobenzidine ([DAB] Vector Laboratories, Burlingame, CA). For IF, Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 568 donkey anti-goat (Invitrogen, Carlsbad, CA) were used as secondary antibodies at 1:1000. Vectastain kit with 4',6-diamidino-2-phenylindole ([DAPI] Vector Laboratories, Burlingame, CA) was used for nuclear staining. All slides were visualized using an Olympus B51 microscope and photographs taken on an Olympus U-TVO.5xc camera (Olympus, Melville, NY) with MagnaFire.

IGFBP-3 ELISA

Detection of secreted levels of IGFBP-3 in rat serum was performed using a mouse/rat IGFBP-3 ELISA kit

(REF E031, Mediagnost Ltd, Reutlingen, Germany) as per the manufacturer's instructions. Blood was collected via heart puncture from rats at time of sacrifice. Blood was then centrifuged, sera collected and frozen at -80°C until further analysis. ELISA plate readings were obtained using a microplate reader emitting a wavelength of 450 nm (Reference filter ≥ 590 nm).

Western blotting

Protein was isolated from liver tissue that had been snap-frozen in liquid nitrogen. Liver lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA) using standard techniques. Antibodies utilized for immunoblotting were as follows: IGFBP-3 at 1:1000 (sc-6004, Santa Cruz Biotechnology), β -Actin at 1:2000 (Ab3280, Abcam, Cambridge, MA), and horseradish peroxidase (HRP)-conjugated IgG antibodies (Santa Cruz Biotechnology) secondary antibodies. ECL plus™ Western blotting detection kit (Amersham Biosciences, Piscataway, NJ) was used to develop the membrane.

Preparation and delivery of IGFBP-3 siRNA

Silencer® In Vivo Ready Pre-Designed siRNAs (Applied Biosystems, Foster City, CA) to rat IGFBP-3 were used for siRNA experiments. Three different siRNAs to IGFBP-3 (AM16831, #199977, #59620, #47753) was administered to each rat to obtain optimal knockdown results. All siRNAs were diluted in sterile phosphate-buffered saline (PBS), and a total siRNA concentration of 200 µM was administered to rats via tail vein injections on days 6 and 8 post-PHX. In addition to receiving IGFBP-3 siRNA, another group of rats (n = 3) received 250 ng/mL of IGFBP-3 protein via tail vein injection on days 6 and 8 post-PHX. As a control, a group of rats (n = 3) received Silencer Select negative Control siRNAs (Applied Biosystems). A total siRNA concentration of 200 µM was administered to control rats via tail vein injections on days 6 and 8 post-PHX. All rats were euthanized at day 9 post-PHX (n = 3 per condition) by injection of sodium pentobarbital (0.1 mL/100 g). All procedures involving animals were approved by the University of Florida IACUC.

Quantitative real-time PCR analysis of mRNA expression

qRT-PCR was performed using predesigned microarray plates containing primers for several genes related to the

(SA Biosciences TGF β bone morphogenetic protein [BMP] Signaling Pathway SuperArray, PARN-035). Sequences for these primers can be obtained from manufacturer's website. Liver samples from experimental animals ($n = 3$ per condition) were pooled and RNA was then isolated from these samples. siRNA isolation was performed as previously described.²¹ cDNA was generated using the SuperScriptTM III First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The standard conditions used for realtime PCR were as follows: 95°C for 10 mins followed by 40 cycles of 15 s denaturation at 95°C, 30 seconds annealing/elongation at 55°C. SYBR[®] Green signal was measured in each step and each sample normalized to β -actin as an internal control. Mean fold gene expression was calculated with SA Biosciences software using the Delta Delta CT method.

Statistical analysis

All results are presented as the means \pm standard deviation (SD), and all data shown were compiled from three separate experiments. Student's *t*-test was used to analyze the differences between two groups. Values were regarded as significant at $*P < 0.05$ and $**P < 0.005$. All error bars represent the SD.

Results

IGFBP-3 protein levels increased in liver and serum during peak days of oval cell proliferation in the rat

Although IGFBP-3 is primarily produced in the liver under normal conditions, changes in expression of IGFBP-3 during oval cell-mediated liver regeneration have never been examined. Real-time polymerase chain reaction (RT-PCR) analysis of cDNA obtained from whole, pooled liver samples from animals that had undergone 2AAF/PHx determined that IGFBP-3 mRNA is in fact present at all time points during this protocol (Figure 1A). To verify expression of IGFBP-3 protein during this phenomenon, Western blot analysis was performed on liver lysate from rats that had undergone 2AAF/PHx (Figure 1B). Analysis of these protein levels in Figure 1C reveals that expression of IGFBP-3, in comparison to normal liver, decreases by approximately 30%, in the days following partial hepatectomy (days 0 to 3). In contrast, IGFBP-3 expression increases by approximately 45% compared to normal IGFBP-3 levels beginning on day 9 of the 2AAF/PHx

protocol (Figure 1C, $P < 0.005$). IGFBP-3 protein levels remained elevated in comparison to the normal rat until day 17, after which IGFBP-3 levels began to decrease and returned to basal levels by day 22. In addition, ELISA analysis showed a similar pattern of increased protein levels in the serum of these same animals. Days 9 and 11 of the 2AAF/PHx protocol had significantly higher serum levels of IGFBP-3 protein, which measured $255 \text{ ng/mL} \pm 45 \text{ ng/mL}$ and $249 \text{ ng/mL} \pm 41 \text{ ng/mL}$ respectively, when compared to IGFBP-3 serum levels of normal, untreated animals which were detected at $150 \text{ ng/mL} \pm 22 \text{ ng/mL}$ (Figure 1D, $P < 0.05$). By day 13, serum levels began to decrease and returned to basal levels by day 22 of the study.

Distribution of IGFBP-3 protein in the liver is altered during oval cell-mediated liver regeneration

The presence of IGFBP-3 protein in the liver was further confirmed histologically through the use of an IGFBP-3 specific antibody. IGFBP-3 expression in the normal liver appeared to be evenly distributed throughout the tissue, as evidenced by the positive signal seen in Figures 2A and 2B. However, on day 9 post-PHx, the distribution and intensity of IGFBP-3 protein expression is altered when compared to the normal liver. Highly positive areas of IGFBP-3 staining can be seen around the central vein (Figures 2C and 2D), in addition to several cells which appear highly positive for IGFBP-3 expression around the periportal region of the liver lobule (Figures 2E and 2F). Similar to results seen in Western blot experiments, IGFBP-3 levels in the liver return to baseline levels by day 22 and appear phenotypically similar to the IGFBP-3 expression pattern found in normal rat liver (Figures 2G and 2H).

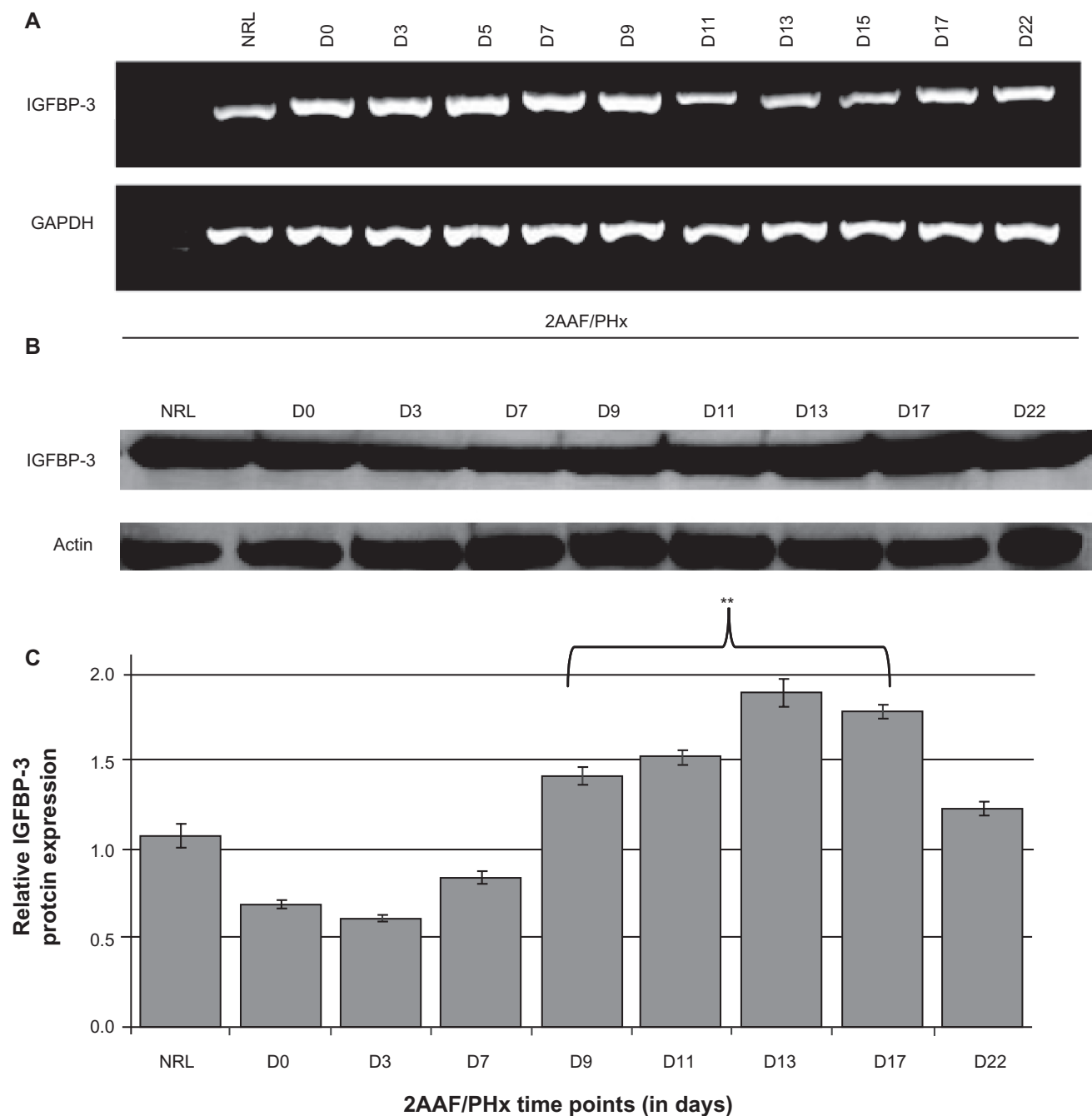
IGFBP-3 induces WB-344 migration while inhibiting IGF-I induced cell proliferation *in vitro*

To determine whether IGFBP-3 could act as a potential chemoattractant and induce oval cell migration we setup a transwell migration assay, utilizing the WB-344 stem-like cell line.^{22,23} WB-344 cell migration was significantly enhanced in all conditions where IGFBP-3 was added to the lower chamber compared to those chambers containing media with FBS alone (Figure 3A, $P < 0.05$).

In addition to promoting cell migration, several groups have reported the effects of IGFBP-3 on either promoting

or inhibiting the proliferative ability of cells both *in vitro* and *in vivo*.^{24–26} To determine what effect IGFBP-3 has on oval cell proliferation, we established several MTT proliferation assays again using WB-344 stem-like cells. Because IGFBP-3 has been shown to mediate many of its effects either independently or dependently on the presence of IGF-I, we included IGF-I at equal concentrations to IGFBP-3. Following exposure to IGF-I at 1 ng/mL for 24 hours, WB-344 cell proliferation

was significantly increased when compared to cells in [BSA] alone. Alternatively, WB-344 cells exposed to IGFBP-3 showed little difference in cell proliferation after 24 hours (Figure 3B). However, when cells were exposed to IGF-I in the presence of IGFBP-3, cellular proliferation was significantly suppressed ($P < 0.05$) as compared to cells treated with IGF-I alone. Following 48 hours of exposure, no proliferative changes could be detected.



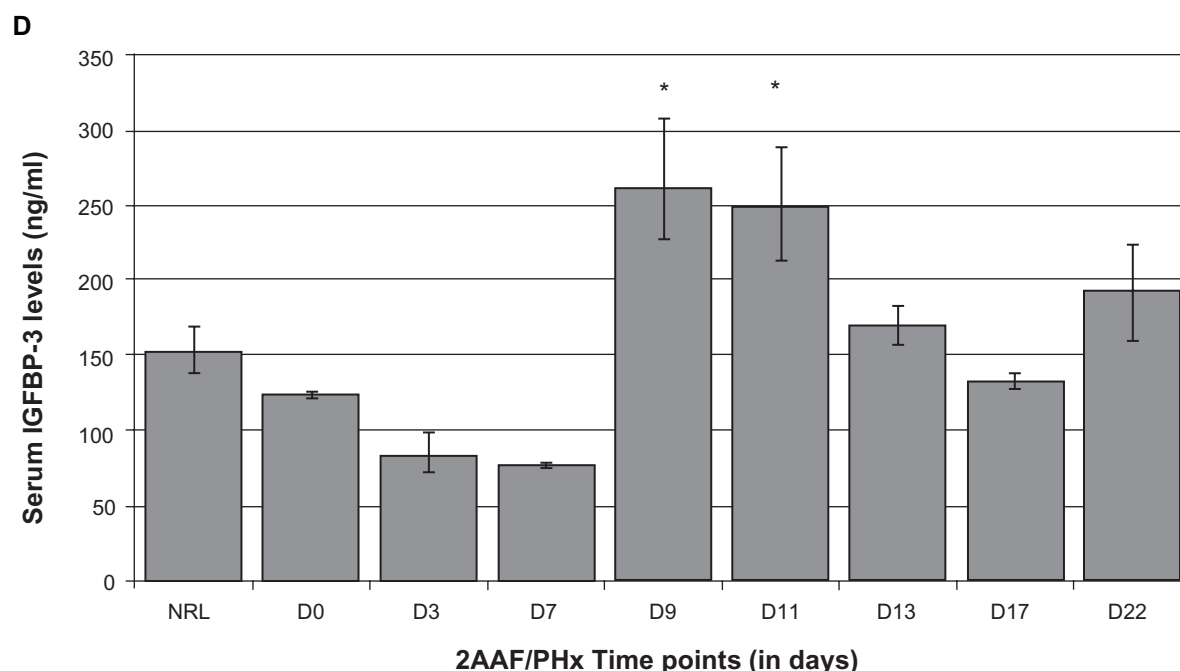


Figure 1 Changes in IGFBP-3 expression during hepatic oval cell activation. **A)** RT-PCR analysis of normal rat liver (NRL) and liver taken at standard 2AAF/PHx time points verifies IGFBP-3 mRNA expression during oval cell-mediated liver regeneration. **B)** Western blot analysis performed on protein isolated from pooled rat liver samples ($n = 3$ per time point) at days 0, 3, 5, 7, 9, 11, 13, 17 and 22 of the 2AAF/PHx protocol and probed for IGFBP-3. Protein from NRL was used as a control. IGFBP-3 was significantly decreased following PHx (day 0), and then increased significantly to levels greater than NRL by day 9. Protein levels remained elevated until day 17 and then decreased to baseline levels by day 22. **C)** Semi-quantitative analysis of IGFBP-3 protein expression. Data shown were compiled from three independent experiments. Expression was normalized to β -actin and significance calculated compared to control animals, $**P < 0.005$. **D)** ELISA analysis for IGFBP-3 expression in serum isolated from animals following 2AAF/PHx. Serum from untreated rats NRL was used as a control. Levels of IGFBP-3 decreased after PHx (day 0) and continued to decrease until day 7 post-PHx. At days 9 and 11, IGFBP-3 levels were significantly increased compared to NRL serum levels. By day 13, serum levels again decreased and returned to baseline levels by day 22. $*P < 0.05$. **Abbreviations:** NRL, normal rat liver; IGFBP-3, insulin-like growth factor binding protein-3; N-2-acetylaminofluorine, (2AAF). PHx, partial hepatectomy.

Addition of exogenous IGFBP-3 corrects for loss of protein expression in siRNA-treated animals

Based on preliminary *in vitro* results suggesting that IGFBP-3 might play a role in the migration and proliferation of oval cells, IGFBP-3 expression during peak oval cell proliferation was suppressed utilizing siRNA technology. In addition to this, we performed a recovery experiment in which animals received IGFBP-3 protein injections in addition to siRNA treatment. Western blot analysis of pooled liver protein at day 9 confirmed a significant loss of IGFBP-3 protein in animals that received IGFBP-3 siRNA (Figure 4A). Semi-quantitative analysis showed an 84% decrease in IGFBP-3 protein when compared to animals that received 2AAF/PHx alone and an 80% decrease when compared to animals that received negative control siRNA (Figure 4B). Additionally, IGFBP-3 expression in animals treated with additional IGFBP-3 was almost equal to that of levels seen in rats treated with 2AAF/PHx alone or those treated with negative control siRNA at day 9 (Figure 4B).

In addition to Western blot analysis, secreted levels of IGFBP-3 were also measured using ELISA techniques. Similar to previous results, secreted IGFBP-3 levels in the serum were again significantly decreased in animals treated with IGFBP-3 siRNA at day 9 (Figure 4C). Animals that received exogenous IGFBP-3 protein in addition to IGFBP-3 siRNA had significantly increased levels of IGFBP-3 levels in their serum compared to animals treated with IGFBP-3 siRNA alone however, the levels were not as high as those detected in animals treated with negative control siRNA or at day 9 post-PHx.

Knockdown of IGFBP-3 leads to increased oval cell proliferation and formation of atypical ductular structures during oval cell activation

H & E staining of liver sections from IGFBP-3 siRNA-treated animals show a dramatic increase in the number of small ovoid cells present at day 9 (Figure 5C) compared to sections from animals that received 2AAF/PHx alone (Figure 5A) or

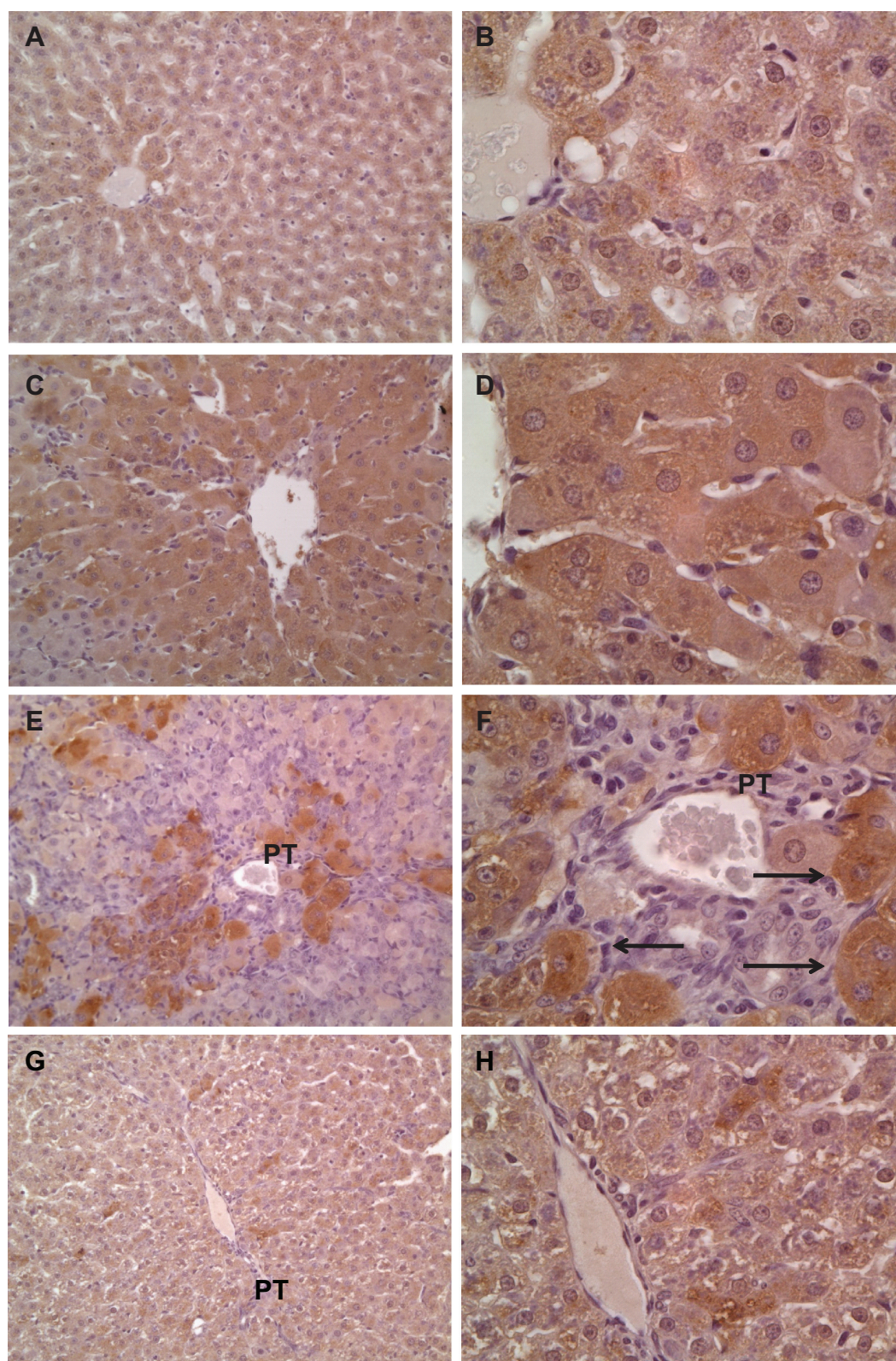


Figure 2 Changes in distribution of IGFBP-3 during hepatic oval cell activation. **A–F**) Immunohistochemical analysis of IGFBP-3 expression in the normal rat liver (A and B). Day 9 post-PHx (C and F) and day 22 post-PHx (G and H). In the normal liver, IGFBP-3 expression is distributed throughout the tissue, with areas of strong signal in the interstitial space between adjacent hepatocytes (A and B). Following 2AAF/PHx, there is an increase in IGFBP-3 expression at day 9 around the central vein (C and D). IGFBP-3 expression can also be found around the portal triad region in day 9 post-PHx tissue, where oval cell infiltration into the regenerating liver is taking place (E and F). Oval cells do not appear to be positive for IGFBP-3 expression. Arrows indicate several cells which are highly positive for IGFBP-3 staining around portal triad region. Magnification: A, C, E, $\times 10$; B, D, F, $\times 20$.

Abbreviations: PT, portal triad; CV, central vein; IGFBP-3, insulin-like growth factor binding protein-3; 2AAF, N-2-acetylaminofluorine; PHx, partial hepatectomy.

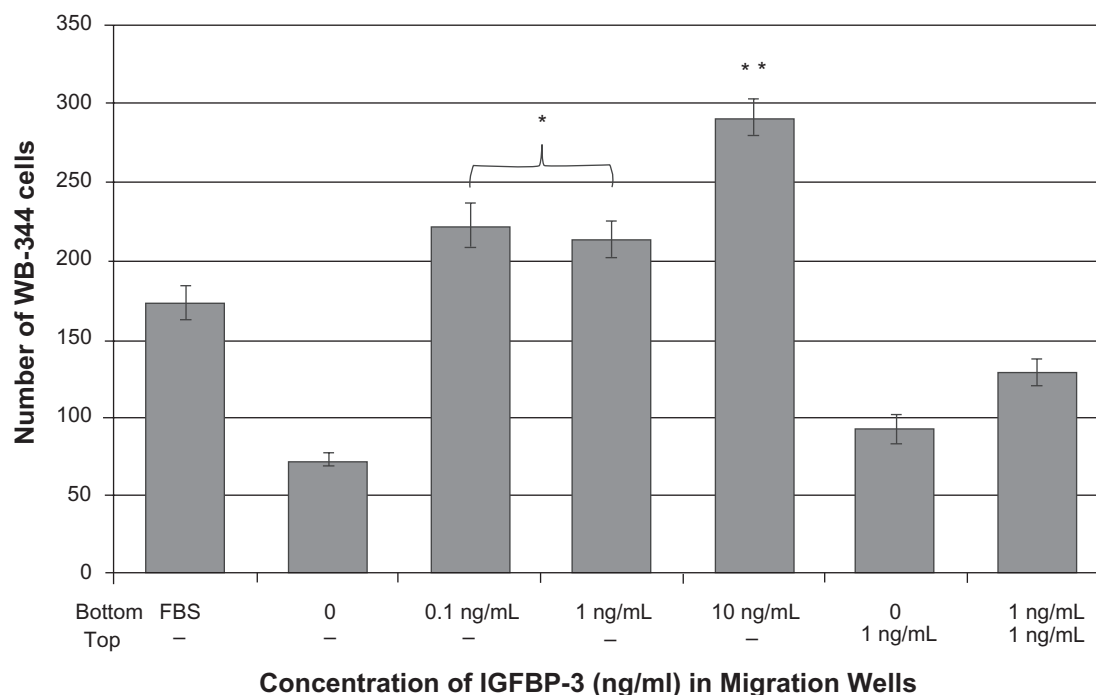
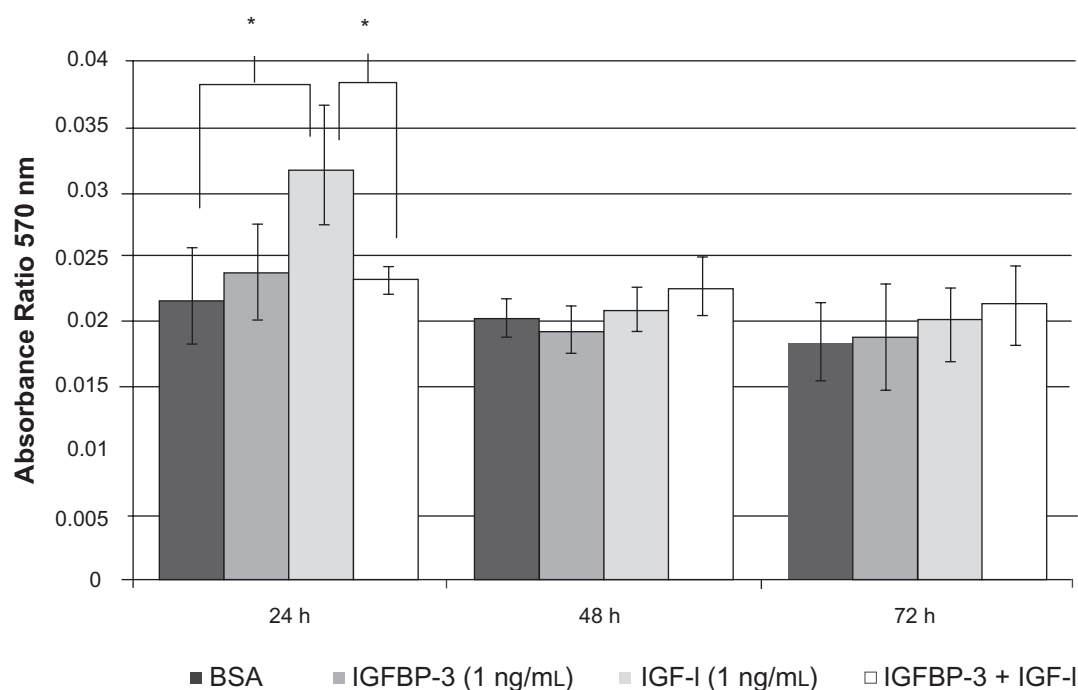
A**B**

Figure 3 IGFBP-3 promotes migration while inhibiting IGF-I induced proliferation of WB-344 cells *in vitro*. **A)** Analysis of transwell migration assays using WB-344 cells. Cells were suspended in serum-free media (containing only 0.5% BSA). Additionally, all experimental wells contained serum free media with the exception of the positive control wells, which contained media which was supplemented with 10% FBS. Various concentrations of IGFBP-3 (0.1, 1, and 10 ng/mL) were added to the bottom wells to analyze chemoattractive effect on cells. WB-344 cell migration increased significantly in the presence of IGFBP-3 with relatively similar effects at 0.1 and 1.0 ng/mL. The most dramatic increase in cell migration was seen when the highest concentration of IGFBP-3 was used (10 ng/mL). **B)** MTT proliferation assays. After 24 h, IGFBP-3 alone had no effect on WB-344 cell proliferation. IGF-I (1 ng/mL) significantly increased WB-344 cell proliferation compared to cells treated with media containing BSA (0.5%) alone. When IGFBP-3 (1 ng/mL) and IGF-I were added together, the proliferative effect of IGF-I was significantly inhibited. Following 48 hours and 72 hours, all conditions showed relatively the same amount of WB-344 cell proliferation. Data shown were compiled from three independent experiments. * $P < 0.05$.

Abbreviations: IGFBP-3, insulin-like growth factor binding protein-3; BSA, bovine serum albumin; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

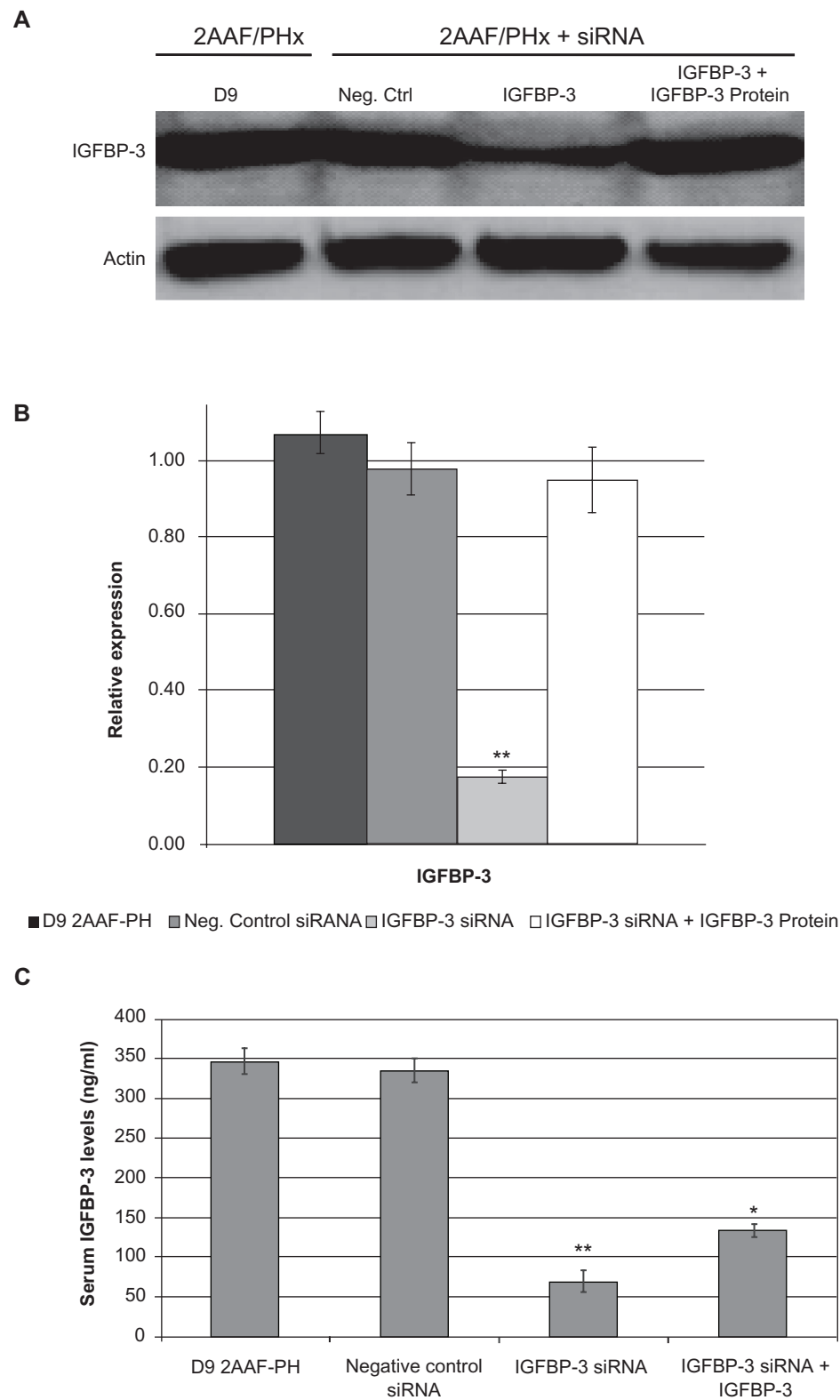


Figure 4 Addition of exogenous IGFBP-3 corrects for loss of protein expression in siRNA-treated animals. **A)** Western blot analysis performed on protein isolated from pooled liver samples ($n = 3$ animals per condition) taken at day 9 post-PHx and probed for IGFBP-3 protein expression. Results show a statistically significant reduction in IGFBP-3 protein expression in IGFBP-3 siRNA-treated animals compared to negative control siRNA and 2AAF/PHx treated animals alone at day 9. Addition of exogenous IGFBP-3 protein resulted in a significant return of IGFBP-3 protein expression to a normal level compared to controls. **B)** Semi-quantitative analysis of IGFBP-3 protein expression. **C.** ELISA results from pooled rat serum taken at day 9 post-PHx. There is a significant reduction in IGFBP-3 secreted protein in animals treated with IGFBP-3 siRNA. Addition of IGFBP-3 protein resulted in a slight increase in secreted IGFBP-3 compared to controls. Data shown were compiled from three independent experiments. Expression was normalized to β -actin and significance calculated by comparison to control animals. ** $P < 0.005$.

Abbreviations: 2AAF, N-2-acetylaminofluorine; PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3.

negative control siRNA (Supplemental Figure 1). IGFBP-3 siRNA-treated livers also had a much higher appearance of cells that were actively undergoing proliferation at day 9, as demonstrated by an increased number of Ki67 positive cells (Figure 5D) compared to controls (Figure 5B and Supplemental Figure 1B). Most noteworthy about IGFBP-3 siRNA-treated livers was the appearance of numerous atypical ductular structures around the portal triad regions (Figure 5E). These structures were not seen in liver sections in animals that received negative control siRNA or in sections from animals that received 2AAF/PHx alone. Ki67 staining of these sections shows that the cells within the duct-like structures are actively undergoing proliferation (Figure 5F).

To further confirm the presence of increased oval cell infiltration in IGFBP-3 siRNA-treated animals, we performed dual immunofluorescent staining utilizing an IGFBP-3 specific antibody and an antibody against OV-6, a known oval cell and biliary cell marker. IGFBP-3 staining of liver sections confirmed the loss of IGFBP-3 protein expression in siRNA-treated livers around the portal triad region of the liver compared to negative control siRNA (Supplemental Figure 2A) and day 9 post-PHx alone (Figure 6, far left panels). IGFBP-3 siRNA-treated livers showed a marked increase in the appearance of OV-6 positive oval cells around the portal triad at day 9 compared to 2AAF/PHx alone at day 9 (Figure 6, middle panels). Merged images of these stains show that the OV-6 positive oval cells are not expressing IGFBP-3 in the liver during oval cell-mediated regeneration (Figure 6, right panels, merge).

Dual Ki67 and OV-6 staining confirms increased oval cell proliferation when IGFBP-3 is suppressed in the liver

To further confirm both the increased presence of oval cells and to demonstrate that these cells are actively undergoing proliferation in the livers of IGFBP-3 siRNA-treated animals, we performed a dual stain for Ki67 and OV-6 expression. Figure 7 demonstrates that IGFBP-3 siRNA-treated animals display both an increase in the number of OV-6 oval cells present in the liver and confirms that nearly every aberrant ductular structure surrounding the periportal region contains cells which are OV-6 positive and Ki67 positive (Figure 7B). These results indicate that the oval cells present in IGFBP-3 siRNA-treated liver are increased in numbers and are actively undergoing proliferation at a rate that is significantly higher than the oval cells present in livers at day 9 post-PHx alone (Figure 7B).

Addition of IGFBP-3 protein inhibits formation of atypical ductular structures and maintains normal liver architecture during oval cell-mediated liver regeneration

As expected, IGFBP-3 staining of livers from animals treated with IGFBP-3 siRNA alone showed a dramatic reduction in IGFBP-3 protein (Figure 8A) as compared to controls (Figure 8C and Supplemental Figure 3A). We also noted the unique appearance of numerous atypical ductular structures in IGFBP-3 siRNA-treated livers that were not present in the negative control siRNA-treated animals or in animals that received 2AAF/PHx alone. Due to the fact that administration of 2AAF inhibits proliferation of the resident hepatocytes in the regenerating liver, it is believed that these atypical ductular structures were derived from the oval cell population. Further histological analysis of these structures revealed that they, like the oval cells, are OV-6 positive (Figure 8B). As expected, the presence of these OV-6 positive ducts could not be found in liver sections from day 9 2AAF/PHx alone (Figure 8D) or in sections from negative control siRNA (Supplemental Figure 3B). However, when IGFBP-3 protein was administered to animals in combination with IGFBP-3 siRNA, we detected an increase in IGFBP-3 staining around the portal triad region of the liver (Figure 8E). In addition to this, the appearance of OV-6 positive atypical ductular structures was no longer detected around the portal triad region (Figure 7F).

Knockdown of IGFBP-3 expression in siRNA-treated animals leads to downregulation of TGF- β family members

Activation of the TGF- β pathway has been associated with IGFBP-3 in a variety of different cell types, including several different types of cancers. Additionally, IGFBP-3 has also been shown to regulate cell proliferation through direct interaction with TGF- β receptors and to latent forms of TGF- β .^{16,27} Because activation of the TGF- β pathway has previously been associated with peak days of oval cell proliferation during specific types of liver regeneration, we asked whether suppression of IGFBP-3 is associated with changes in the expression of proteins associated with the TGF- β family. Quantitative RT-PCR analysis confirmed that IGFBP-3 siRNA-treated animals had reduced IGFBP-3 expression, with a more than 9-fold reduction in IGFBP-3

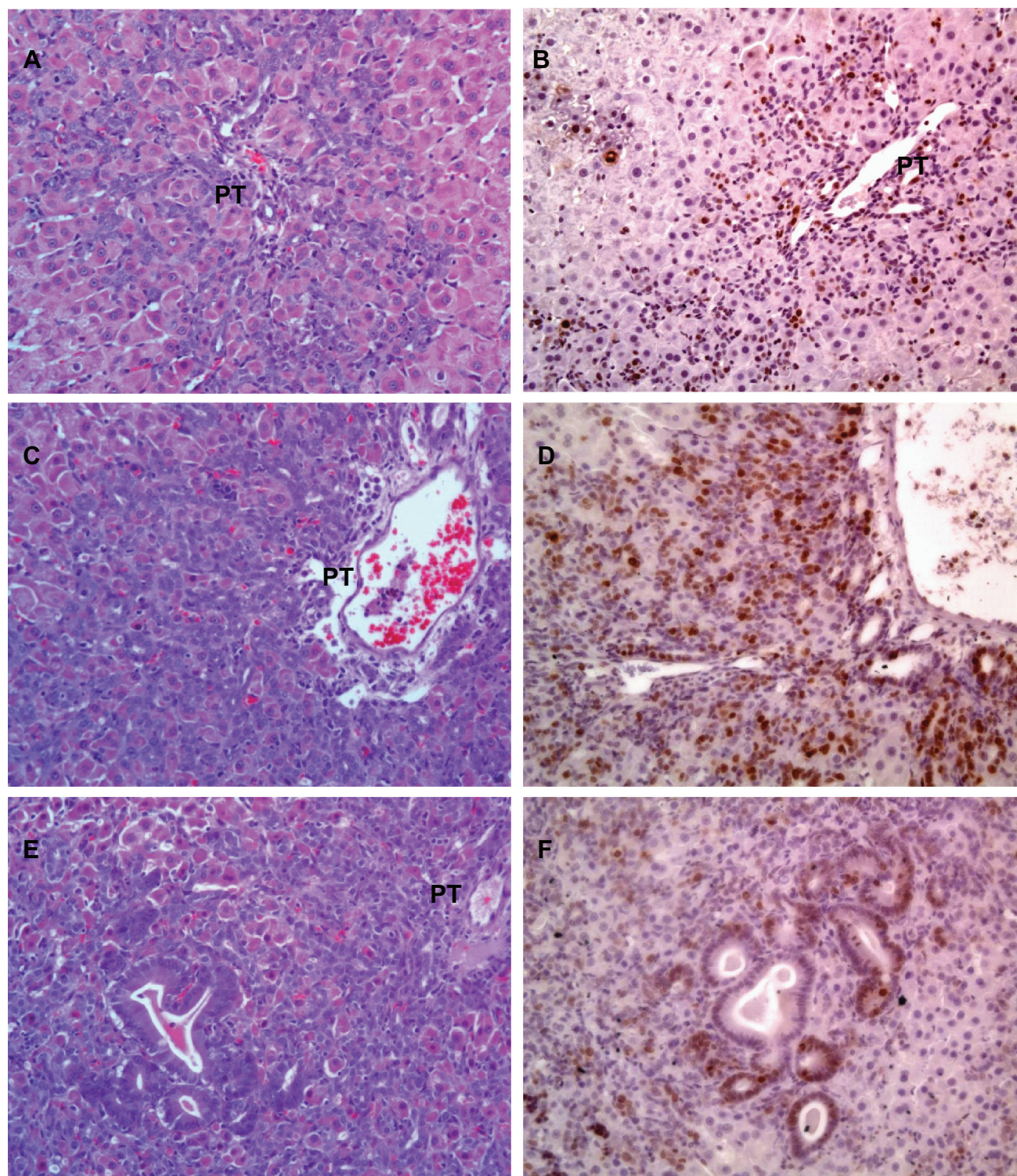


Figure 5 Knockdown of IGFBP-3 leads to increased oval cell proliferation and formation of atypical ductular structures during oval cell activation. **A)** Representative H & E staining of a day 9 post-PHx paraffin liver section. **B)** Ki67 staining of a day 9 post-PHx paraffin liver section. **C)** Representative H & E staining of IGFBP-3 siRNA-treated liver at day 9. Note the increased appearance of small ovoid cells around the portal triad region of the liver. **D)** Ki67 staining of IGFBP-3 siRNA-treated liver at day 9. The number of cells that are actively undergoing proliferation are increased compared to day 9 post-PHx alone. **E)** Representative H & E stain of an atypical ductular structure found in IGFBP-3 siRNA-treated animals at day 9 post-PHx. **F)** Ki67 staining of livers treated with IGFBP-3 in addition to IGFBP-3 siRNA. Magnification: A to F $\times 20$.

Abbreviations: H & E, hematoxylin and eosin; PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3.

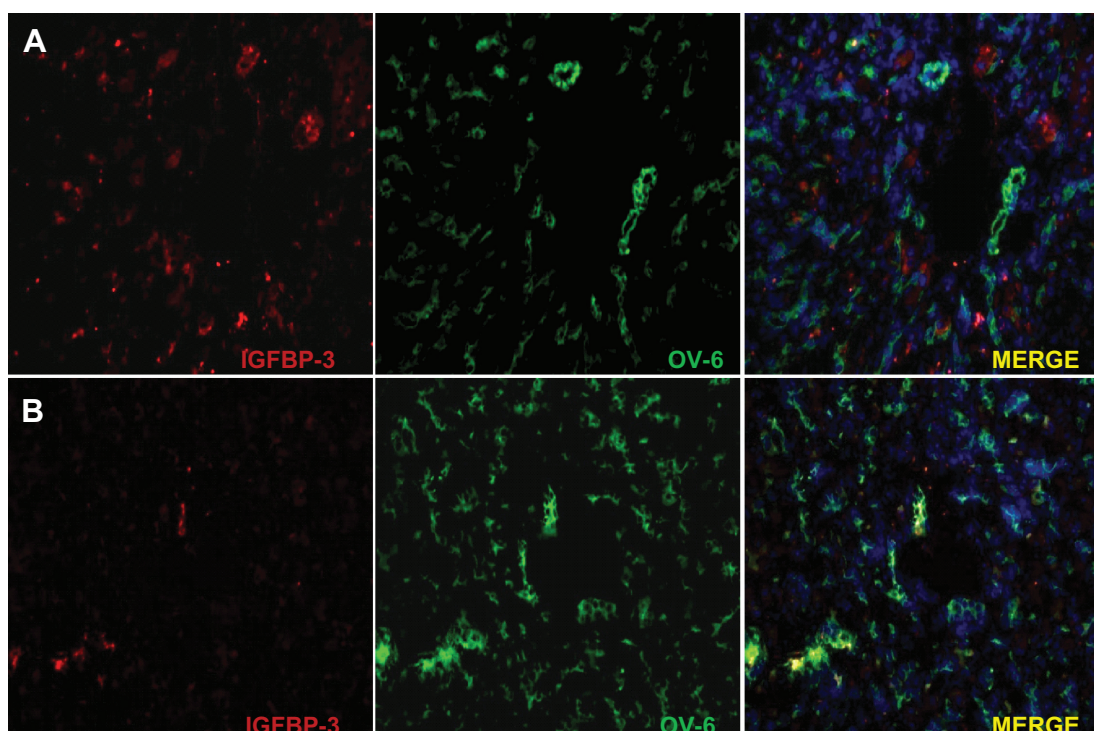


Figure 6 Knockdown of IGFBP-3 during oval cell-mediated liver regeneration corresponds to increase in oval cell infiltration. **A)** Dual IF staining of a day 9 liver following 2AAF/PHx alone. **B.** day 9 liver section following 2AAF/PHx with IGFBP-3 siRNA treatment. IGFBP-3 is expressed by cells around the portal triad region of the liver (A, left). Activation of oval cells at this time is confirmed by the presence of OV-6 staining (A, middle). Dual staining demonstrates that the OV-6 positive oval cells do not express IGFBP-3 (A, merge). IGFBP-3 staining confirms loss of protein expression due to IGFBP-3 siRNA treatment (B, left) compared to 2AAF/PHx alone. OV-6 staining shows an increase in the number of positive oval cells around the portal triad region in livers treated with IGFBP-3 siRNA (B, middle). Magnification: A and B $\times 20$.

Abbreviations: 2AAF, N-2-acetylaminofluorine. PHx, partial hepatectomy. IGFBP-3, insulin-like growth factor binding protein-3.

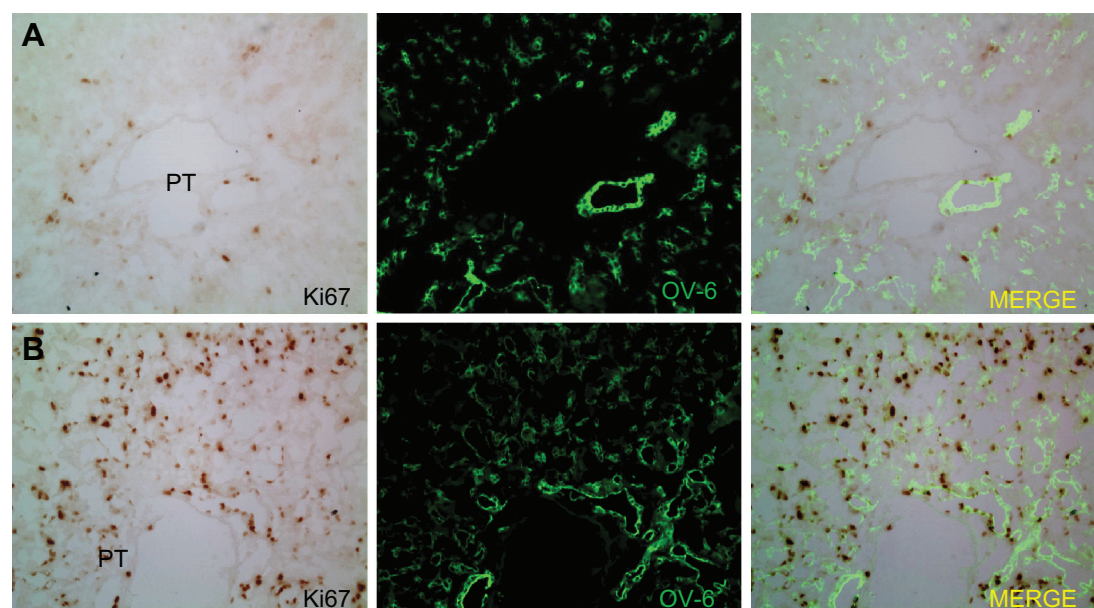


Figure 7 Dual Ki67 and OV-6 staining confirms increased oval cell proliferation when IGFBP-3 is suppressed in the liver. **A)** Dual staining of a day 9 liver following 2AAF/PHx alone. **B)** IGFBP-3 siRNA-treated liver section at day 9 post-PHx. Cells undergoing proliferation were visualized through the use of a Ki67 antibody, followed by incubated with a biotinylated secondary antibody and developed using DAB (A and B, left). Activation of oval cells at this time is confirmed by the presence of OV-6 staining (A and B, middle) shown in green through the use of a FITC-conjugated secondary antibody. Merged images confirm that livers treated with IGFBP-3 siRNA contains a greater number of oval cells which are undergoing proliferation in comparison to livers at day 9 following PHx alone. Magnification: A and B $\times 20$.

Abbreviations: 2AAF, N-2-acetylaminofluorine; PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3; DAB, 3,3'-diaminobenzidine.

mRNA compared to controls (Figure 9A). Animals treated with additional IGFBP-3 protein showed a 3-fold decrease in IGFBP-3 mRNA. A significant reduction in IGF-I mRNA expression was also detected in animals treated with IGFBP-3 siRNA alone or with additional IGFBP-3 protein (>8-fold and >7-fold downregulation, respectively). This suggests that the effects mediated by IGFBP-3 during oval cell activation may be IGF-1 dependent and that when IGFBP-3 is lost due to siRNA, IGF-1 expression decreases as well. Interestingly, mRNA levels of the proto-oncogene *myc* were significantly increased following siRNA treatment (Figure 9A). This suggests that IGFBP-3 may play a role in regulating cell proliferation in the liver during oval cell activation.

Although no differences in TGF- β 1 mRNA expression was detected, we noted a significant fold reduction (>7-fold decrease) in mRNA expression of TGF- β R2 in IGFBP-3 siRNA-treated animals (Figure 9B). Addition of exogenous IGFBP-3 protein appeared to correct somewhat for this loss however, TGF- β R2 mRNA was still significantly reduced in comparison to the controls at day 9. In addition, mRNA expression of Smad 2, 3, and 4 were also significantly downregulated in IGFBP-3 siRNA-treated animals. These results indicate that IGFBP-3 mediated effects in the liver may somehow involve activation of the TGF- β R2 and many downstream targets of the TGF- β pathway including Smads 2–4.

Discussion

In this study, we demonstrated that IGFBP-3 expression in the rat is elevated during known days of peak oval cell proliferation in the liver and in the serum. Additionally, *in vitro* studies using the WB-344 oval-like cell line determined that exposure to exogenous IGFBP-3 protein at various concentrations enhanced transwell cell migration. This suggests that IGFBP-3 acts as a potent chemoattractant *in vitro*, with the highest number of migrating cells found in the chambers that contained the highest concentration of IGFBP-3 (10 ng/mL). Additionally, due to the fact that all wells contained a serum free medium (with the exception of the positive control wells which contained FBS), we believe that the effect of IGFBP-3 on WB-344 migration may be independent of IGF-I. In addition to this, initial RT-PCR analysis of WB-344 cells determined that the cells do not express IGF-I mRNA (data not shown). Therefore, the effects seen on WB-344 migration in the presence of IGFBP-3 appear to be IGF-I dependent, as IGF-I was not present in the media or produced by the WB-344 cells. These results, taken with IHC results showing the unequal distribution of IGFBP-3 protein in the liver

at day 9 post-PHx, suggest that IGFBP-3 may promote oval cell migration across a concentration gradient. We believe that infiltrating oval cells from the periportal region, where IGFBP-3 concentration is relatively low, migrate toward the central vein, where IGFBP-3 concentrations are much greater. When IGFBP-3 is lost due to siRNA treatment, oval cell migration is inhibited, thus leading to a “backup” of oval cells around the periportal region.

In addition to our results, several other groups have suggested IGFBP-3 as a potent chemoattractant for stem cells and progenitor cells both *in vitro* and *in vivo*. In a study by Lofqvist and colleagues, administration of exogenous IGFBP-3 protein enhanced CD34+ endothelial progenitor cell migration by 30% to injured areas of the retina compared to IGFBP-3 null mice following oxygen induced blood vessel damage.²⁸ This increase in epithelial progenitor cell presence was also shown to contribute to increased blood vessel repair and re-growth in these animals. Similarly, in a study by Chang and colleagues, IGFBP-3 was found not only to induce hematopoietic stem cell migration *in vitro*, but was also found to contribute to endothelial progenitor cell proliferation and differentiation.¹² These studies support our findings that IGFBP-3 can act as a potent chemoattractant for stem cell populations. These results further suggest that IGFBP-3 may enhance stem cell migration following acute tissue injury, such as that caused by 70% partial hepatectomy.

In addition to increased cell migration, we also found that IGFBP-3 inhibited IGF-I induced proliferation of WB-344 cells, as seen in our MTT proliferation assays. Based on the known functions of IGFBP-3, this phenomenon suggests that IGFBP-3 may be binding to free IGF-I in the culture medium, thus inhibiting IGF-I from promoting cell proliferation *in vitro*. Based upon the similarities of oval cells and WB-344 cells, these results would suggest that hepatic oval cells are responsive to IGF-I and that the effects mediated by IGFBP-3 in regards to oval cell proliferation may be IGF-I dependent, whereas IGFBP-3 induced migration appears to be an IGF-I independent function.

When IGFBP-3 siRNA was used in combination with the 2AAF/PHx protocol, the number of proliferating oval cells present in these livers was elevated compared to animals at day 9 post-PHx alone. This is evidenced by a dramatic increase in the number of OV-6 positive and Ki67 positive cells around the periportal regions of the liver lobule in siRNA-treated animals compared to animals at day 9 post-PHx alone. Most notably however about these IGFBP-3 siRNA-treated livers was the appearance of numerous atypical ductular structures that were actively

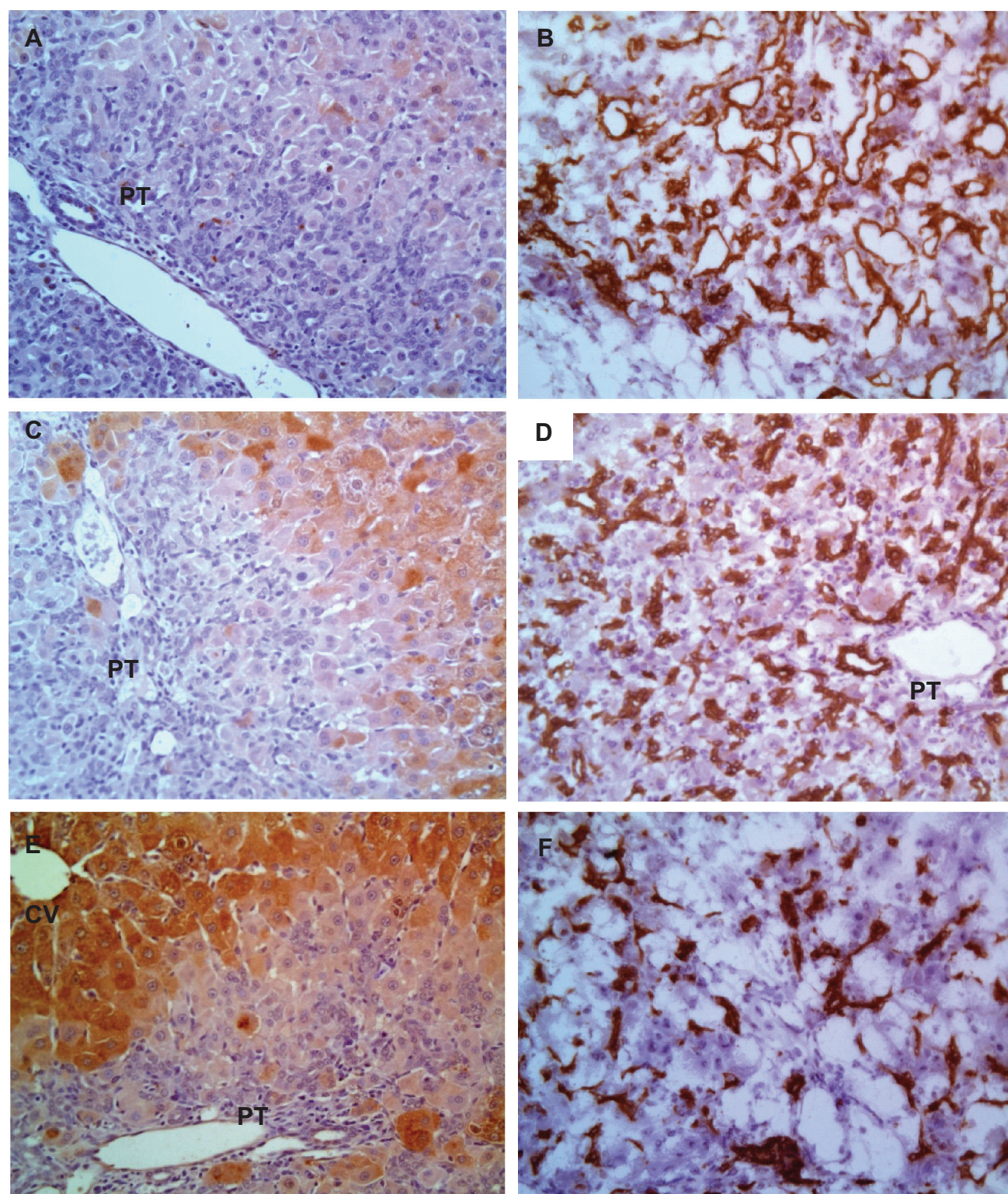


Figure 8 Addition of IGFBP-3 protein inhibits formation of atypical ductular structures and maintains normal liver architecture during oval cell-mediated liver regeneration. **A,C,E** Immunohistochemical analysis of IGFBP-3 expression in IGFBP-3 siRNA at day 9 (A), day 9 post-PHx alone (C), and IGFBP-3 siRNA plus IGFBP-3 protein-treated animals (E). In IGFBP-3 siRNA-treated liver, there is a significant loss of IGFBP-3 expression in the cells surrounding the portal triad region compared to day 9 post-PHx alone (C). **B** OV-6 staining of frozen liver sections taken from siRNA animals shows a phenotypic change in arrangement of oval cells at day 9. The oval cells are arranged in numerous ductular formations. **D** OV-6 staining of liver at day 9 post-PHx. **F** OV-6 staining of liver taken from animals that received IGFBP-3 protein in addition to IGFBP-3 siRNA. Note the reduction in the number of atypical ductular structures found in these sections. Magnification: A to E $\times 20$.

Abbreviations: 2AAF, N-2-acetylaminofluorine; PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3.

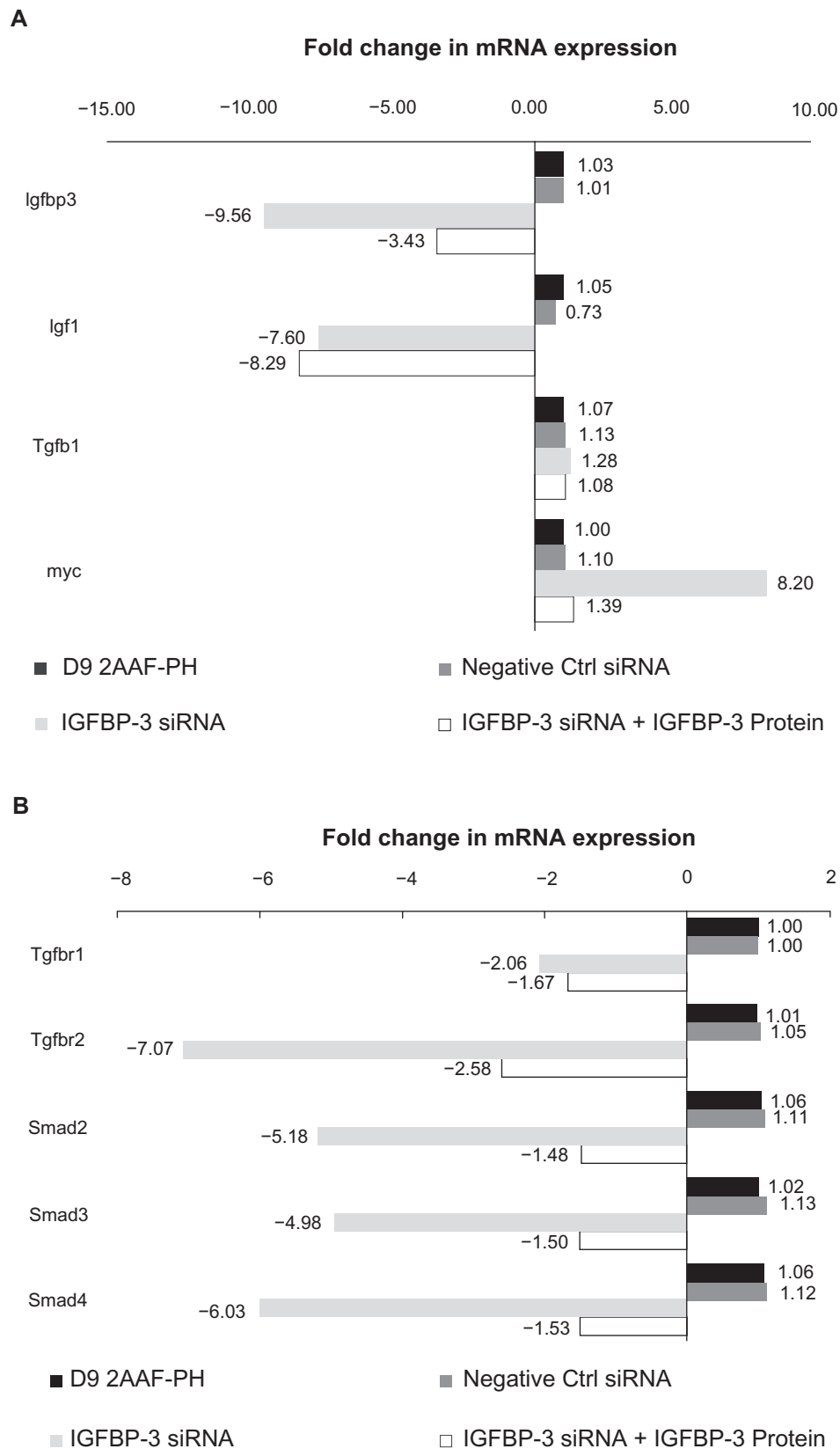


Figure 9 Knockdown of IGFBP-3 expression in siRNA-treated animals leads to downregulation of TGF- β family members. **A** and **B**. qRT-PCR analysis of the TGF- β BMP signaling pathway. IGFBP-3 siRNA-treated animals and animals treated with additional protein showed a significant reduction in *Igfbp3* mRNA and *Igf1* mRNA. *Myc*, a proto-oncogene, was significantly upregulated in IGFBP-3 siRNA-treated animals (A).

Abbreviations: IGFBP-3, insulin-like growth factor binding protein-3; TGF- β , transforming growth factor- β ; qRT-PCR, quantitative real time polymerase chain reaction; BMP, bone morphogenetic protein.

undergoing proliferation which were not present in the livers of animals treated with 2AAF/PHx alone or with negative control siRNA. Although day 9 post-PHx is considered the peak of oval cell proliferation, we found that when IGFBP-3 expression in the liver is suppressed we observed an even higher level of oval cell proliferation the liver, which is essentially causing the oval cells to differentiate improperly during the process of liver regeneration into these aberrant ductular structures. Due to the fact that administration of 2AAF inhibits proliferation of the hepatocytes during this specific type of liver regeneration in the rat, it is further evidence that these atypical ductular structures are derived from the oval cell population. Therefore, we believe that IGFBP-3 plays a role in oval cell differentiation during liver regeneration and that when IGFBP-3 expression is lost, oval cells lose the ability to differentiate toward the hepatocyte lineage. When IGFBP-3 was reintroduced to these animals, the appearance of these atypical ductular formations was greatly reduced, further confirming that IGFBP-3 may play a role in the ability of these cells to differentiate toward the hepatocyte lineage. These results demonstrate that the loss of IGFBP-3 expression during peak oval cell activation leads to an over-proliferation of oval cells and the appearance of these atypical ductular structures that are believed to arise from the oval cell population. This further suggests that IGFBP-3 may play a regulatory role in the ability of these cells to undergo controlled proliferation and may also affect their ability to differentiate toward the hepatocyte lineage *in vivo*.

Although reduced expression of IGFBP-3 protein during oval cell-mediated liver regeneration leads to an increased presence of oval cells at day 9 post-PHx, these cells do not appear to be functioning properly to aid regeneration of the organ. This is evidenced both by their tendency to favor differentiation toward bile ductular structures rather than basophilic hepatocytes and by evidence of reduced liver weights compared to overall body weight at day 9 post-PHx in the absence of IGFBP-3 (Supplemental Figure 4). Under normal conditions of oval cell-mediated liver regeneration, the average liver weight at day 9 post-PHx was 6.4 g, or approximately 3.3% of the animal's total body mass. However, animals treated with siRNA had significantly lower liver weights at day 9 post-PHx, weighing in at an average of 4.4 g, or approximately 2.3% of total body mass. The overall reduction in liver weight in siRNA-treated animals compared to day 9 post-PHx alone suggests that oval cell function and contribution to liver regeneration was impaired, most likely due to their inability to differentiate toward the hepatocyte lineage, resulting from reduced IGFBP-3 expression in the

liver. Overall, addition of exogenous IGFBP-3 protein to siRNA-treated animals appeared to have a varied effect on the liver weights of these animals in comparison to total body mass, indicating that oval cell function may still be slightly improved in some of these livers.

In an effort to identify a signaling pathway through which IGFBP-3 regulated oval cell processes during liver regeneration, qRT-PCR analysis was performed using microarray plates which contained several genes involved in activating and regulating the TGF- β superfamily. Analysis of these experiments determined that animals treated with IGFBP-3 siRNA during oval cell activation displayed decreased mRNA expression of several key components of the TGF- β family, including Smads 2-4 and TGF- β RII. The TGF- β pathway has been shown to play a role in IGFBP-3 production where several groups have shown that TGF- β 1 and TGF- β 2 enhance IGFBP-3 mRNA and protein expression.^{15,29,30} Studies have also shown that IGFBP-3 can stimulate the phosphorylation of the TGF- β type I receptor (TGF- β RI) leading to phosphorylation of downstream transcription factors including Smads 2 and 3.^{25,31} These studies support our findings in that IGFBP-3 is able to mediate its effects through interactions with the TGF- β receptors and the Smad proteins. However, further analysis needs to be conducted to determine if the phosphorylation of the proteins is altered in our experimental conditions.

As previously stated, oval cells are bipotential in nature and have the ability to become either hepatocytes or biliary cells under normal regenerative conditions in the liver. Therefore, when the ability of these oval cells to differentiate toward the hepatocyte lineage is inhibited, the oval cells favor differentiation toward the default biliary pathway. Additional studies in our lab uncovered similar findings in livers of rats treated with Wnt1 shRNA following 2AAF/PHx. When Wnt1 expression was suppressed, oval cells were inhibited from differentiating toward the hepatocyte lineage. As with treatment with IGFBP-3 siRNA, oval cells were found to be undergoing atypical ductular hyperplasia, indicating that these cells had defaulted to a bile duct phenotype. Similar to IGFBP-3 expression during oval cell-mediated liver regeneration, Wnt1 protein expression is also increased during peak days of oval cell activation.

Both IGFBP-3 and members of the Wnt family have previously been implicated in the formation of several different types of cancer in humans and in rodents. In a study by Declercq and colleagues, members of the Wnt signaling pathway and IGF pathway, including IGFBP-3, were found to be upregulated in pleomorphic adenomas of the salivary

glands in mice.³² Additionally, a link between IGFBP-3, Notch and Wnt signaling was identified in a study of prostate cancers containing a dysregulation in the homeobox gene, HOXC6.³³ Here, increased expression of HOXC6 in prostate tumors was found to directly inhibit IGFBP-3 expression. In addition to this, epigenetic silencing of Wnt-suppressing target genes in these tumors led to HOXC6 activation of Notch signaling.³³ These studies suggest a possible connection between IGFBP-3, the Wnt signaling pathway and Notch pathway in regards to regulating cell proliferation and cancer progression.

IGFBP-3 has been shown to play a role in regulating cellular growth, either in the presence of IGF-I or through IGF-I independent mechanisms, in a variety of cell and tissue types.^{26,34} Additionally, decreased levels of IGFBP-3 either in tissue or in serum, have been associated with an increase in cell proliferation in a variety of cancers in humans.^{35,36} Results of these experiments support our current findings when IGFBP-3 siRNA was used during oval cell activation. Here, we found that when IGFBP-3 expression in the liver was suppressed, cell proliferation was increased as evident by an increased number of Ki67-positive cells around the periportal region during oval cell-mediated liver regeneration. We therefore believe that IGFBP-3 acts as a negative regulator of oval cell proliferation, and that when IGFBP-3 expression in the liver is suppressed we see a dramatic increase in oval cell numbers compared to animals at day 9 post-PHx alone, resulting in a impairment of these cells to differentiate and function properly during the process of oval-cell mediated liver regeneration.

Decreased IGFBP-3 levels in cancers have also been associated with increased invasiveness and tumor aggressiveness. In a clinical study of patients with ovarian endometrioid carcinoma, low IGFBP-3 expression correlated with a higher tumor grade, more advanced tumor stage and overall lowered survival rate compared to patients with normal or elevated IGFBP-3 levels.^{37,38} Again, these results suggest that IGFBP-3 may play an important role in regulating cell proliferation and may act as an invasion-metastasis suppressor in certain types of cancer.

It has been reported that oval cells may be capable of participating in the formation of certain types of cancer in the liver, including hepatocellular carcinoma (HCC).³⁹ In a study by Gong and colleagues, it was found that mRNA expression of IGFBP-3, in addition to both IGFBP-1 and 4, were significantly downregulated in patients with HCC compared to patients with normal or cirrhotic livers.⁴⁰ In addition to this, a clinical study of HCC patients with cirrhosis

found that IGFBP-3 mRNA levels in tumor tissue was significantly lower compared to IGFBP-3 mRNA levels in the surrounding nontumor liver tissues and in comparison to normal liver.⁴¹ Additionally, Ki67 staining of these tumors and adjacent nontumor tissues from these patients demonstrated an increase in cell proliferation when IGFBP-3 mRNA levels were decreased when compared to normal tissue.⁴¹ These results further demonstrate that IGFBP-3 may play a role in regulating proliferation of cells in the liver and supports our theory that loss of IGFBP-3 expression in the liver leads to aberrant proliferation of the oval cells.

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Disclosures

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Supplementary material

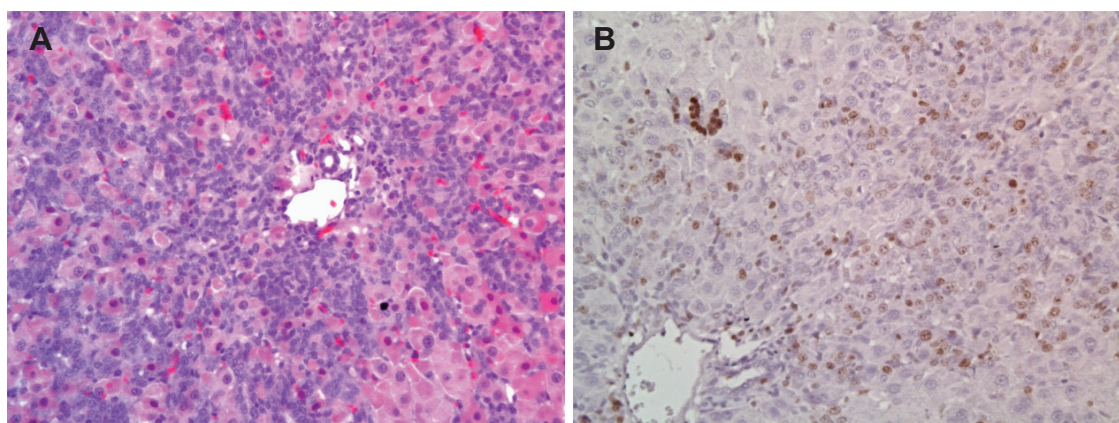


Figure S1 Knockdown of IGFBP-3 leads to increased oval cell proliferation and formation of atypical ductular structures during oval cell activation. **A)** Representative H & E staining of a day 9 post-PHx paraffin liver section from an animal treated with negative control siRNA. **B)** Representative Ki67 staining of a day 9 post-PHx paraffin liver section from an animal treated with negative control siRNA. Magnification: A and B $\times 20$.

Abbreviations: H & E, hematoxylin and eosin; PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3.

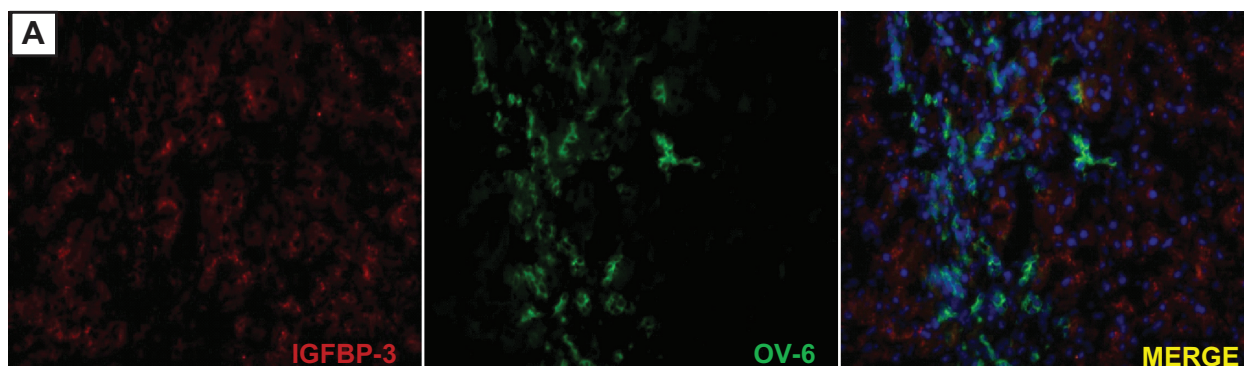


Figure S2 Knockdown of IGFBP-3 during oval cell-mediated liver regeneration corresponds to increase in oval cell infiltration. **A)** Dual IF of a negative control siRNA-treated liver. IGFBP-3 is expressed by cells around the portal triad region of the liver (A, left). Activation of oval cells at this time is confirmed by the presence of OV-6 staining (A, middle). Dual staining demonstrates that the OV-6-positive oval cells do not express IGFBP-3 (A, merge).

Abbreviation: IGFBP-3, insulin-like growth factor binding protein-3.

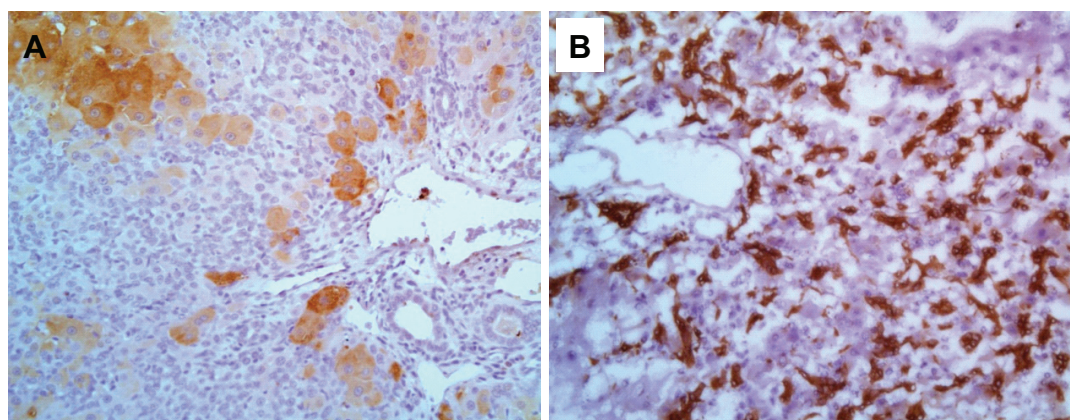


Figure S3 Addition of IGFBP-3 protein inhibits formation of atypical ductular structures and maintains normal liver architecture during oval cell-mediated liver regeneration. **A)** IGFBP-3 staining of a negative control siRNA-treated liver. Note the appearance of several IGFBP-3-positive cells around the portal triad region where oval cells are seen entering into the liver. **B)** OV-6 staining of a negative control siRNA-treated liver. Note the lack of OV-6-positive ducts around the portal triad. Magnification: A and B $\times 20$.

Abbreviation: IGFBP-3, insulin-like growth factor binding protein-3.

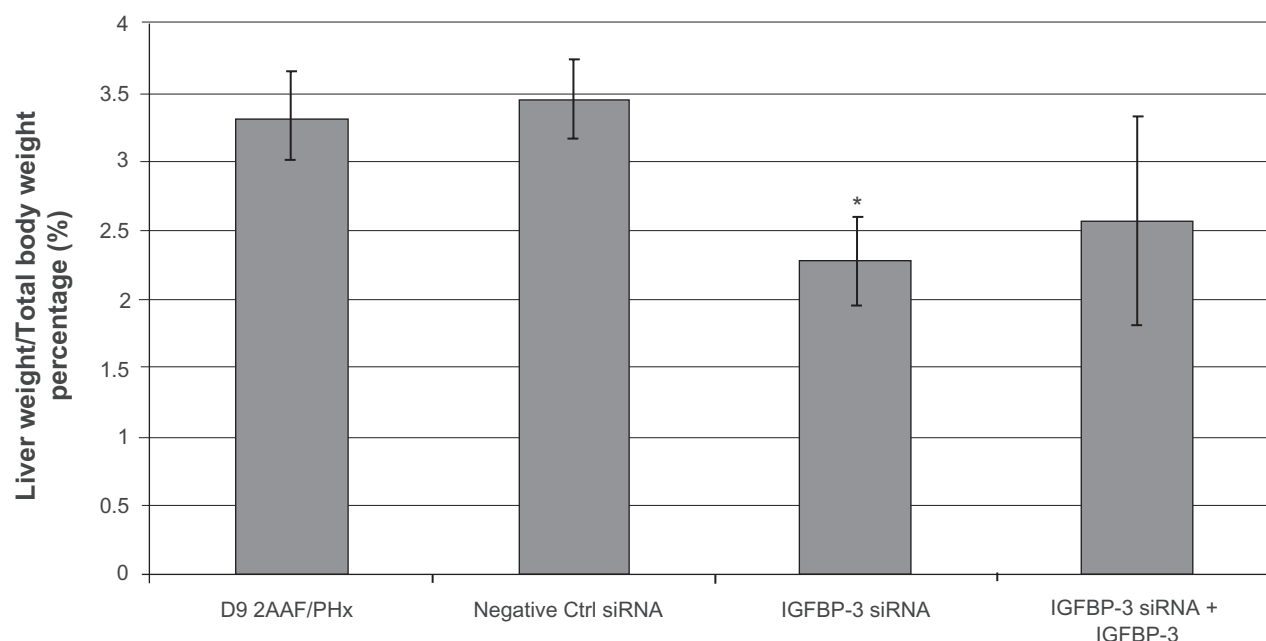


Figure S4 Knockdown of IGFBP-3 protein during oval cell-mediated liver regeneration resulted in reduced liver weights in comparison to overall body weight at day 9 post-PHx. Animals treated with IGFBP-3 siRNA had significantly lower liver weights at day 9 post-PHx compared to animals at day 9 post-PHx alone or animals treated with negative control siRNA at day 9 post-PHx. Addition of exogenous IGFBP-3 protein resulted in varied liver weights at day 9 post-PHx. Data shown are the averages of three animals per condition. * $P < 0.05$.

Abbreviations: PHx, partial hepatectomy; IGFBP-3, insulin-like growth factor binding protein-3.

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