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PTHrP/PTHR1 and TGF-β Levels Are Inversely Associated in Liver Regeneration

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Abstract

Background. Transforming growth factor (TGF)-β provides growth control in liver regeneration. We have recently demonstrated that TGF-β induced parathyroid hormone-related protein (PTHrP) expression and secretion, and PTHrP mediated TGF-β-induced apoptosis in liver cells. However, whether PTHrP signaling pathway is altered during liver regeneration is unknown. Therefore we used a murine hepatectomy model in this study and tested the hypothesis that both PTHrP and TGF-β signaling pathways are upregulated during liver regeneration.

Methods. Swiss Webster mice received 70% hepatectomy or sham operation and euthanized at different time points post-surgery for analyses. Liver regeneration was determined by liver/body weight and proliferating cell nuclear antigen (PCNA) staining. mRNA levels of TGF-β1, TGF-β receptors, PTHrP, and PTHrP receptor 1 (PTHR1) were measured by real-time quantitative PCR. Protein levels of TGF-β1 were measured by ELISA and PTHrP and PTHR1 were measured by Western blotting.

Results. After 70% hepatectomy, the liver regeneration began at 24 hours and was restored to 82% of original liver mass at day 7. TGF-β1 and its receptor levels increased at 24 and 48 hours after hepatectomy, while PTHrP levels decreased at 12 hours and PTHR1 levels decreased at 12, 24 and 48 hours after hepatectomy. The levels of these molecules returned to similar levels as that in sham animals thereafter.

Conclusion. We demonstrated that an upregulation of the TGF-β and its receptors were associated with a down-regulation of PTHrP/PTHR1 expression during liver regeneration, which may contribute to hepatocyte proliferation and regeneration after hepatectomy.

Key words: TGF-β; Parathyroid hormone-related protein; Liver regeneration

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INTRODUCTION

Liver regeneration after hepatic tissue loss is a fundamental parameter of liver response. The main causes for the loss include liver resection in hepatocellular carcinomas and trauma, or liver damage due to viral infections, chemicals and alcohol intoxication.(Fausto, 2004; Fausto & Riehle, 2005) Many growth factors and cytokines are involved in orchestrating this regenerative process. For instance, tumor necrosis factor (TNF)-α and interleukin-6 have been implicated in the initial step of rendering hepatocytes in a state of replicate competence. As a following step, hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α stimulate hepatocyte replication. Finally, TGF-β and activin suppress cell growth and terminate liver regeneration at a set point. (Michalopoulos & DeFrances, 1997; Shimizu et al., 2001)

TGF-β is a multifunctional protein with a broad spectrum of cellular activities ranging from regulation of target gene activity to the control of cell growth and apoptosis. TGF-β exerts its biological effects through a cell surface receptor complex, the TGF-β type I (TβRI) and type II receptors (TβRII). Upon ligand binding, TβRII phosphorylates TβRI, which subsequently activates the canonical pathway by phosphorylating Smad2 and Smad3. Phosphorylated Smad2 and Smad3 form a heteromeric complex with Smad4. The complex translocates into the nucleus, and regulates transcription of target genes, including plasminogen activator inhibitor-1, type I collagen, the cyclin-dependent-kinase inhibitors p15 and p21.(Heldin, Miyazono, & ten Dijke, 1997; Massague, 1998) TGF-β mRNA presence is low in normal liver cells, but increases significantly within a few hours after partial hepatectomy.(Braun et al., 1988) Mechanisms underlying the early increased TGF-β are still unclear, probably due to release of the bound TGF-β from matrix in response to perturbations of the matrix. TGF-β in the liver regulates parenchymal, inflammatory cells and hepatic stellate cells. The early increased TGF-β may be required for new matrix synthesis as hepatic histology becomes rearranged during and after regeneration.(Michalopoulos & DeFrances, 1997) TGF-β also has immunomodulatory properties. By in large, TGF-β is considered a specific candidate for termination of liver regeneration due to its inhibitory effects. TGF-β inhibits hepatocyte proliferation and induces apoptosis, thus serves as an important negative control limiting the regenerative process in response to partial hepatectomy.(Breitkopf, Godoy, Ciuclan, Singer, & Dooley, 2006; Karkampouna, Ten Dijke, Dooley, & Julio, 2012) Inhibition of TGF-β signaling augments liver regeneration.(Liska et al., 2012; Zhong et al., 2010)

Parathyroid hormone-related protein (PTHrP) is a polyprotein derived from normal and malignant cells. The complex growth factor-like properties of PTHrP display a variety of effects including the regulation of cell differentiation, proliferation and death.(Philbrick et al., 1996) In contrast to the situation in humoral hypercalcemia of malignancy in which PTHrP plays the role of a classical "endocrine" hormone, PTHrP plays predominantly paracrine and/or autocrine roles under normal circumstances. However, accumulating evidence suggests that PTHrP may in fact act more like a cytokine than a classic hormone, being widely expressed in normal tissues by a variety of cell types, where it has local autocrine, paracrine, or intracrine effects.(Philbrick et al., 1996)

Previous studies demonstrated that TGF-β increases PTHrP expression by specifically up-regulating transcription from the PTHrP P3 promoter through Smad3/Ets1 synergism,(Lindemann, Ballschmieter, Nordheim, & Dittmer, 2001) and enhances PTHrP secretion through Smad and p38 MAP kinase pathways. (Kakonen et al., 2002) Neutralization of PTHrP secreted by HepG2 liver cells resulted in increased cell growth, suggesting that PTHrP can function as an autocrine or paracrine growth factor to suppress the growth of the liver cells.(Li, Seitz, Selvanayagam, Rajaraman, & Cooper, 1996) Recently, we reported that TGF-β induces PTHrP expression in hepatocytes *in vitro*, knockdown of PTHrP gene expression or neutralization of secreted PTHrP isoforms blocks TGF-β-induced apoptosis. Therefore, PTHrP functions as a novel mediator for TGF-β-induced apoptosis in liver cells.(Cao et al., 2013) Since TGF-β signaling pathway is activated in liver regeneration, we attempted to examine *in vivo* whether there is a correlation between levels of TGF-β and PTHrP in a hepatectomy model mimicking the process of liver regeneration.

METHODS

Animals and e**xperimental design.** Swiss Webster mice (male, 6-8 weeks) were purchased from Harlan (Indianapolis, IN). The mice were randomized to receive either 70% hepatectomy or sham operation. (Greene & Puder, 2003; Jackson et al., 2008) The mice were euthanized over a time course after operation at 12, 24, 48, 72, and 168 hour (n=5 mice/group/time point), liver tissues was harvested, the liver and total body weight were recorded, portions of the liver were removed for analysis. All animal studies were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. **Immunohistochemistry analysis (IHC)** was performed using a monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (DAKO) as described. (Gao et al., 2014; Gao et al., 2013; Murray et al., 1999) **Real-time quantitative (q) PCR** was performed using Taqman probe sets of mouse TGF-β1, TGF-β receptor I (TβRI) and II (TβRII), PTHrP and PTHrP receptor 1 (PTHR1) as described.(Cao et al., 2007) **TGF-β1 protein levels** were measured using TGF-β1 LINCOplex kit (LINCO Research, St. Charles, MO). **Western blotting analysis** was performed using antibodies against PTHrP (Santa Cruz Bio Inc.) and PTHR1 (EMD Millipore Calbiochem) as described. (Cao et al., 2013) **Statistical analysis.** Data were expressed as mean±SEM. Differences between groups at the indicated time points were analyzed by *t*-test and $p<0.05$ is considered significant.

RESULTS

Liver regeneration after partial hepatectomy. To validate the liver regeneration model following hepatectomy, the mice were randomized to receive either 70% hepatectomy or sham operation. Following

hepatectomy, a gradual increase in remnant liver weight was noted in mice. Remnant liver was 2.2% at 12 hours, 2.7% at 24 hours, 3.7% at 48 hours, 4.2% at 72 hours, and 4.4% by day 7 of body weight. The liver weight restored to 82% compared to sham group by day 7 (Figure 1A). To confirm liver regeneration, IHC was performed utilizing PCNA antibody, a marker of cell proliferation, on liver tissue sections. The PCNA staining increased at 48 hours in hepatectomy group, followed by a peak staining at 72 hours and returned to a similar level as that in sham animals by day 7 (Figure 1B).

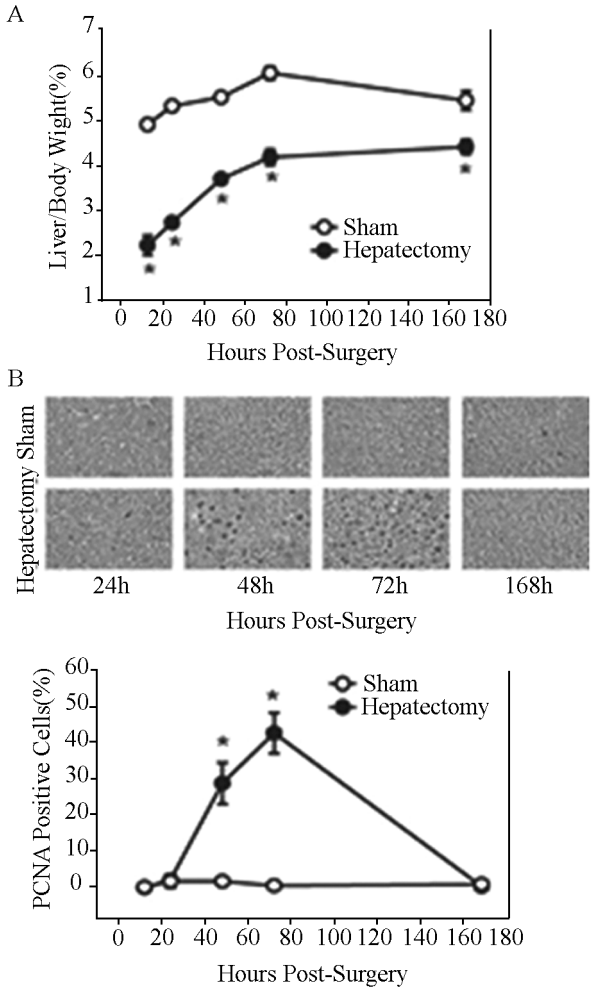

Figure 1
Liver regeneration

Swiss Webster mice were randomized to either 70% hepatectomy (n=5) or sham operation (*n*=5). **A.** Mice were sacrificed over a time course after operation, and the wet remnant liver weight as a percent of body weight was determined as an estimation of liver regeneration. Data are expressed as mean±SEM. **B.** Liver tissue sections stained with PCNA were examined at high power (200X), and 20 non-overlapped fields were evaluated. Nuclear labeling indices for PCNA were determined by counting at least 1,000 hepatocyte nuclei. Data are expressed as mean±SEM. Arrows point to the representative PCNA positive staining. *p<.05 compared to sham group.

Upregulation of TGF-β and its receptor during liver regeneration. TGF-β signaling molecules were assessed following hepatectomy using qPCR for TGF-β1, TβRI and TβRII mRNA expression in liver tissue, and TGF-β1 protein levels in sera. In the hepatectomy group, TGF-β1 mRNA expression in the liver increased at 24 and 48 hours post-resection compared to the sham controls, then returned to a similar level as that in sham controls after 72 hours (Figure 2A); TGF-β1 protein levels increased in the sera at 24 hours post-resection in the hepatectomy group (Figure 2B). Furthermore, T*βRI mRNA* expression increased at 24 and 48 hours (Figure 3A); TβRII mRNA expression increased at 48 hours post-resection in the hepatectomy group compared to the sham group (Figure 3B). These results demonstrate components of the TGF-β signaling pathway are elevated, suggesting that TGF-β signaling pathway is activated during liver regeneration.

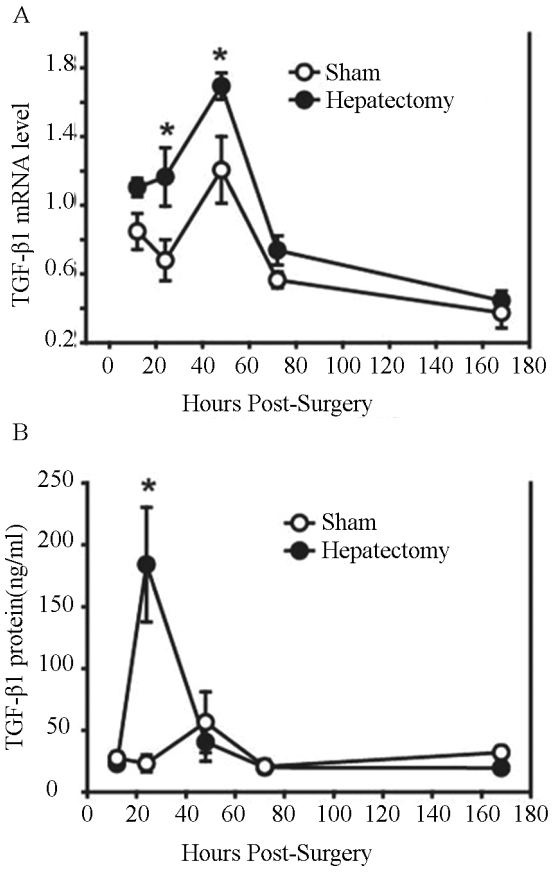

Figure 2
TGF-β Expression Increased During Liver Regeneration

The mouse liver samples were collected as in Figure 1. **A**. qPCR was performed on total RNA isolated from

the liver samples to determine TGF-β1 mRNA level. **B**. ELISA was used to quantitate TGF-β1 protein level in the mouse sera. Data are expressed as mean±SEM. *p<.05 compared to sham group.

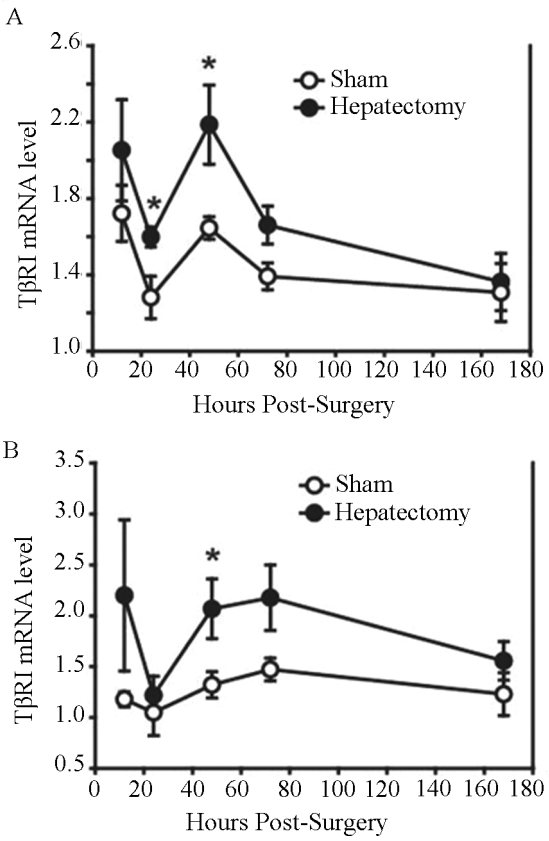

Figure 3
TGF-β Receptor Expression Increased During Liver Regeneration

The mouse liver samples were collected as in Figure 1. qPCR was performed on RNA isolated from liver to determine TβRI (**A**), and TβRII (**B**) mRNA levels. Data are expressed as mean±SEM. *p<.05 compared to sham group.

Downregulation of PTHrP and PTHR1 during liver regeneration. PTHrP is induced in rat liver during endotoxemia and stimulates the hepatic acute phase response, while the PTHR1 mRNA is downregulated during this proces. (Funk, Moser, Grunfeld, & Feingold, 1997). To determine whether PTHrP signaling pathway is altered in liver regeneration, PTHrP and PTHR1 mRNA and protein levels were evaluated in mouse liver tissue after hepatectomy. Compared to sham controls, PTHrP mRNA levels decreased at 12 hours (Figure 4A), and PTHR1 mRNA levels decreased at 12, 24 and 48 hours (Figure 5A), post-resection in the hepatectomy group. Both PTHrP (Figure 4B) and PTHR1 (Figure 5B) protein levels significantly decreased at 12 hours post-resection in the hepatectomy group compared to the sham controls. Taken together, these results suggest that PTHrP signaling pathway is downregulated during hepatic regeneration.

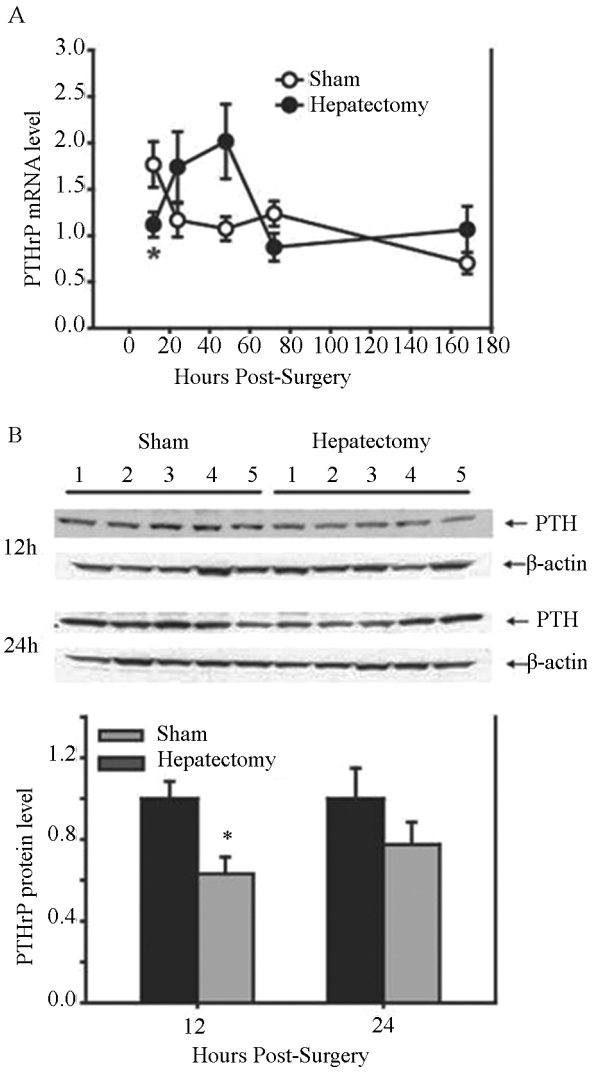

Figure 4
PTHrP Expression Decreased During Liver Regeneration

The mouse liver samples were collected as in Figure 1. **A.** qPCR was performed on RNA isolated from liver to determine PTHrP mRNA levels. **B.** Western blotting analysis was used to determine PTHrP protein levels. β-actin served as a protein loading control. PTHrP protein signals were quantified and normalized against β-actin. Data are expressed as mean±SEM. *p<.05 compared to sham group.

The mouse liver samples were collected as in Figure 1. **A.** qPCR was performed on RNA isolated from liver to determine PTHR1 mRNA levels. **B.** Western blotting analysis was used to determine PTHR1 protein levels. β-actin served as a protein loading control. PTHR1 protein signals were quantified and normalized against β-actin. Data are expressed as mean±SEM. *p<.05 compared to sham group.

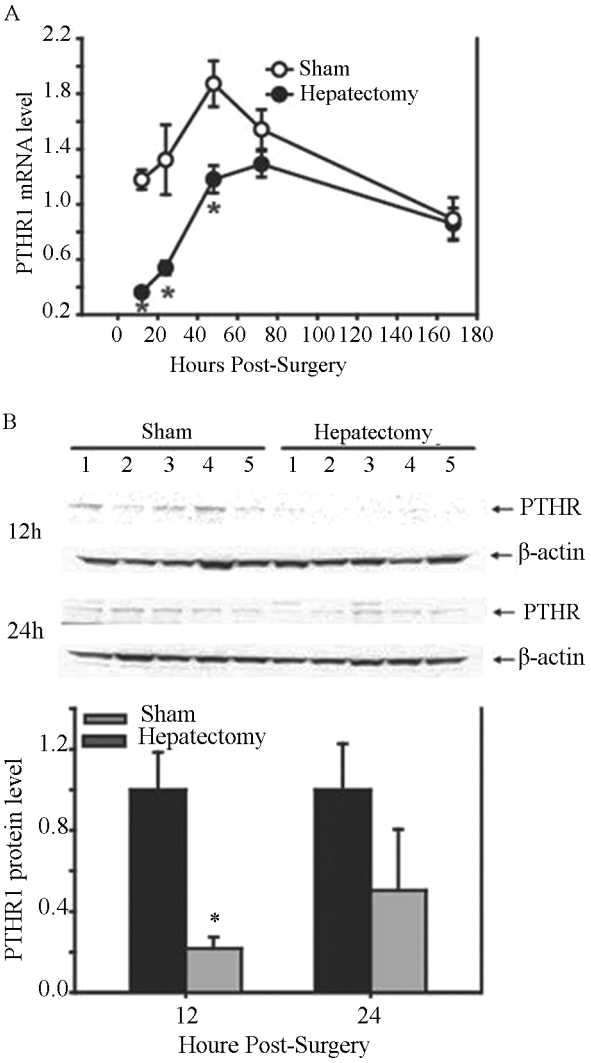

Figure 5
PTHR1 Expression Decreased During Liver Regeneration

The mouse liver samples were collected as in Figure 1. **A.** qPCR was performed on RNA isolated from liver to determine PTHR1 mRNA levels. **B.** Western blotting analysis was used to determine PTHR1 protein levels. β-actin served as a protein loading control. PTHR1 protein signals were quantified and normalized against β-actin. Data are expressed as mean±SEM. *$p<.05$ compared to sham group.

DISCUSSION

The mechanisms involved in hepatocyte replication and liver regeneration following hepatic tissue loss are not fully understood. Growth factors and cytokines appear to play important roles at various stages of this tightly regulated process. The present study demonstrated that the expression of TGF-β1 and its receptors increased during liver regeneration, suggesting the activation of TGF-β signaling pathway. On the other hand, levels of PTHrP and its receptor decreased in the mouse liver after partial hepatectomy, indicating a down-regulation of PTHrP signaling pathway.

In animal model of liver regeneration, after a rapid phase of hepatic growth and restructuring, liver regeneration eventually ceases. DNA synthesis is mostly completed by 72 hours. TGF-β1, a known inhibitor of proliferation in hepatocyte cell lines, is a logical candidate to stop the excess replicative and regenerative process.(Michalopoulos & DeFrances, 1997) Expression of TGF-β1 was shown to be increased during liver regeneration induced by a 70% partial hepatectomy (Bissell, Wang, Jarnagin, & Roll, 1995; Jakowlew et al., 1991). After partial hepatectomy, an early peak of TGF-β2 and -β3 was present in all four cell types, including hepatocytes, sinusoidal endothelial cells, Kupffer cells (liver macrophages), and lipocytes (Ito or stellate cells), followed by a sustained increase in mRNA for TGF-β1, -β2, and -β3 primarily in the hepatocyte population (Bissell et al., 1995). Our study showed a similar expression pattern of TGF-β1 after hepatectomy (Figure 2); however, almost at the same time, liver regeneration was occurring (Fig. 1). It was observed that hepatocytes isolated from regenerating liver from 12 to 48 hours after hepatectomy were resistant to TGF-β1 mito-inhibitory effects (Houck & Michalopoulos, 1989), which is an important phenomenon because it may allow hepatocytes to proliferate even though concentrations of TGF-β1 are increasing. However, the underlying mechanism responsible for this resistance is largely unknown.

Partial hepatectomy and the hepatotoxin carbon tetrachloride (CCl_4) are often used experimental models for liver regeneration study. In both models, TGF-β pathway is activated, and hepatocytes proliferate despite the presence of increased TGF-β. Different from partial hepatectomy, CC14 model is associated with tissue injury and inflammation. Karkampouna's group demonstrates that co-administration of a TβRI inhibitor and CCl_4 promote liver regeneration in mice.(Karkampouna, Goumans, Ten Dijke, Dooley, & Kruithof-de Julio, 2015) CCl_4 specifically damages hepatocytes located around the central veins, and the TβRI inhibitor targets hepatocytes without affecting other cell types. Thus the findings provide important evidence on the specific roles of TGF-β in liver regeneration by targeting hepatocytes, and support TGF-β as a candidate to terminate liver regeneration. Although hepatocytes are parenchymal cells in liver, under liver damage or loss, TGF-β is produced by and acts on multiple cells for its diverse functions. Its role in other cell types deserves further investigation.

As reported previously, PTHrP mediated TGF-β-induced growth inhibition in HepG2 liver cells,(Li et al., 1996) and TGF-β-induced apoptosis in Hep3B and HuH-7 liver cells.(Cao et al., 2013) The *in vivo* study of PTHrP in mouse liver using endotoxemia model demonstrated that PTHrP mRNA expression was induced and PTHR1 expression was downregulated, providing evidence for

the regulated expression of PTHrP in adult liver, and suggesting that PTHrP may be one additional member of the cytokine/growth factor cascade produced locally in liver that can act to stimulate the hepatic acute phase response.(Funk et al., 1997) Considering liver as a whole organ including multiple cell types, in comparison with *in vitro* studies in hepatocytes, we speculate that cell-type specific responses may well exist, what was observed is the net outcome from responses of mixed cell types.

In the current mouse model, the decreased PTHrP and PTHR1 in the earlier stage of regeneration may be due to suppression by unidentified mechanisms, rendering hepatocytes resistant to TGF-β's growth inhibitory effects. This is in line with the observation that hepatocytes isolated from regenerating liver from 12 to 48 hours after hepatectomy are resistant to TGF-β mito-inhibitory effects (Houck & Michalopoulos, 1989). Thus, hepatocytes proliferate in the presence of increased TGF-β. In addition, TGF-β levels are not significantly elevated at time points earlier than 24 h, indicating regulation of PTHrP/PTH1R independent of TGF-β at these earlier stages. We also speculate that in later stages, the suppression mechanisms on PTHrP/PTHR1 diminish. At these later time points, TGF-β levels are significantly elevated, with consequent regulation of the PTHrP/PTH1R pathway. TGF-β and PTHrP signaling pathways therefore reconnect, leading to growth arrest of hepatocytes and stop of regeneration. Therefore, our current study has advanced our *in vitro* findings (Cao et al., 2013) to an *in vivo* hepatectomy model and suggests a potential link between TGF-β and PTHrP signaling pathways that may contribute to hepatocyte proliferation and regeneration.

Opposing effects of TGF-β and PTH have been reported in bone remodeling. Phosphorylation of PTH1R by TβRII leads to downregulation of PTH signaling, while the recruitment of TβRII to PTH1R by PTH dampens TGF-β signaling (Qiu et al., 2010). Thus TGF-β and PTHrP signaling may work both directions to regulate each other's function in a cell or organ-specific context.

Understanding molecular and cellular mechanisms in liver regeneration will facilitate translation of experimental data into new treatment strategies, as clinical human partial liver transplantation continues to rise. Once the isolation and storage of human hepatocytes and intrahepatic stem cells are successfully developed, cell transplantation might be useful in patients with acute liver failure.(Fausto & Riehle, 2005)

As a future scope, the relationship between TGF-β and PTHrP can be cross-examined by liver-specific knockout of TGF-β or PTHrP signaling molecules in animals for functional and signaling analysis. The *in vivo* approach can be coupled with *in vitro* knockdown in multiple cell types for detailed mechanistic studies.

In conclusion, we have shown that the upregulation of TGF-β and the receptor levels are associated with the down-regulation of PTHrP/PTHR1 expression during liver regeneration. Our data suggest that the down-regulation of PTHrP signaling pathway concomitant with the increased TGF-β may contribute to hepatocyte proliferation and regeneration. Therefore, PTHrP is proposed here as a new member of the cytokine/growth factor cascade regulating liver regeneration.

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