# Proliferation-inhibiting pathways in liver regeneration (Review)

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Received March 25, 2016; Accepted March 13, 2017

DOI: 10.3892/mmr.2017.6613

**Abstract.** Liver regeneration, an orchestrated process, is the primary compensatory mechanism following liver injury caused by various factors. The process of liver regeneration consists of three stages: Initiation, proliferation and termination. Proliferation-promoting factors, which stimulate the recovery of mitosis in quiescent hepatocytes, are essential in the initiation and proliferation steps of liver regeneration. Proliferation-promoting factors act as the 'motor' of liver regeneration, whereas proliferation inhibitors arrest cell proliferation when the remnant liver reaches a suitable size. Certain proliferation inhibitors are also expressed and activated in the first two steps of liver regeneration. Anti-proliferation factors, acting as a 'brake', control the speed of proliferation and determine the terminal point of liver regeneration. Furthermore, anti-proliferation factors function as a 'steering-wheel', ensuring that the regeneration process proceeds in the right direction by preventing proliferation in the wrong direction, as occurs in oncogenesis. Therefore, proliferation inhibitors to ensure safe and stable liver regeneration are as important as proliferation-promoting factors. Cytokines, including transforming growth factor-β and interleukin-1, and tumor suppressor genes, including p53 and p21, are important members of the proliferation inhibitor family in liver regeneration. Certain anti-proliferation factors are involved in the process of gene expression and protein modification. The suppression of liver regeneration led by metabolism, hormone activity and pathological performance have been reviewed previously. However, less is known regarding the proliferation inhibitors of liver regeneration and further investigations are required. Detailed information regarding the majority of known anti-proliferation signaling pathways also remains fragmented. The present review aimed to understand the signalling pathways that inhbit proliferation in the process of liver regeneration.

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*Key words:* liver injury, liver regeneration, proliferation-inhibiting, oncogenesis, liver disease

**Contents**



## 1. Introduction

The liver, an organ with a complex structure and function, is damaged by various factors, including viruses, drugs, trauma, chemotoxicity and metabolic disorders. A reduction in liver mass and ischemia-reperfusion caused by surgery are also causes of liver injury in patients with diseases of the liver. The high regenerative potential is the primary mechanism by which the liver compensates for loss of weight and assists in recovery from injury. Reasonable control of liver regeneration is of substantial value in therapy for liver diseases.

There have been studies on liver regeneration for over a century with notable progress. The mechanisms of liver regeneration have been elucidated in detail and have benefited from the contribution of partial hepatectomy (PH) models, first established by Higgins and Anderson in 1931. The classic process of liver regeneration, which involves numerous genes, consists of three stages: Initiation, proliferation and termination (1). Hepatocytes leave their quiescent state, resulting in DNA synthesis, with the stimulation of priming signals, including interleukin (IL)-6, tumor necrosis factor-α (TNF-α) and nitric oxide in the initiation step. The proliferation step is characterized by hepatocytes entering the G1 phase of the cell cycle, and mitogens, including hepatocyte growth factor (HGF), transforming growth factor (TGF)-α and epidermal growth factor (EGF), are essential in this step. When the remnant liver grows to a suitable volume, similar to that of the original liver, regeneration enters the termination step, during which stop and differentiation signals are involved. Compared with the initiation and proliferation steps, the termination step is less well understood.

As is already known, the liver regeneration process is a well-orchestrated process requiring a balance between pro-proliferation and anti-proliferation factors. However, the majority of studies on liver regeneration have been associated with the regulation of initiation and proliferation-promoting signals, with less known regarding termination and growth inhibitors. The proliferation inhibitors act as a 'brake' and 'steering wheel' to control the speed and direction of liver regeneration. The remnant hepatocytes in the damaged liver do not stop growing to the correct size and the proliferation is not suppressed. In extreme cases, the proliferation of liver cells may lead to oncogenesis. For example, loss of the expression of suppressor of cytokine signaling (SOCS)3 enhances liver regeneration but allows hepatocellular carcinoma formation (2). Furthermore, certain growth inhibitors can themselves function as tumor suppressor genes, for example p53 and p21, which are essential for the inhibition of carcinogenesis from liver damage by chronic injury (3). The known proliferation-inhibiting factors and associated pathways are discussed in this review.

## 2. Cytokine-associated pathways

*TGF-β.* TGF-β is the most well-known hepatocyte proliferation inhibitor and stop signal in the process of hepatic regeneration. There have been investigations on the association between TGF and liver regeneration for >30 years. Nakamura *et al* reported in 1985 that TGF-β from platelets inhibited the DNA synthesis of adult rat hepatocytes induced by epidermal growth factor (EGF) in primary culture (4). The administration of platelet-derived TGF-β has also been shown to suppress liver regeneration in a two-thirds PH rat model (5). The inhibition of TGF-β1 with monoclonal antibody has been shown to enhance liver regeneration in a porcine model of partial portal vein ligation (6). A previous study also found that inhibiting the TGF-β pathway with the TGF-β type I receptor kinase inhibitor increased hepatocyte proliferation during acute liver damage (7).

Previous investigations demonstrated that TGF-β is predominantly secreted by nonparenchymal cells, including hepatic stellate cells (HSCs), Kupffer cells (KCs) and platelets, in liver regeneration and modulates the proliferation of hepatocytes in a paracrine manner (8,9). Subsequent studies reported that TGF-β can also be expressed in regenerating hepatocytes (10,11). The expression of TGF-β was found to increase at 4 h and reached a peak 72 h following PH when DNA synthesis had stopped, suggesting that TGF-β was involved in the inhibition and termination of liver regeneration (8,12). Further evidence for the anti-proliferation function of TGF-β1 includes the reduction in DNA synthesis in transgenic mice with overexpressed TGF-β1 following PH (13). Although several members are included in the TGF-β superfamily, the β1 form is the most important subtype in regeneration (10,12).

TGF-β modulates the proliferation of hepatocytes through multiple mechanisms. The earliest mechanism to be identified is the inhibition of EGF-induced DNA synthesis by TGF-β following its binding to TGF-β receptors on the surface of hepatocytes (8,11,14). Investigations found that TGF-β receptors were single-pass transmembrane serine/threonine kinases. When the TGF-β ligand binds to the complex of TGF-β receptors, the receptor-regulated cytoplasmic small mothers against decpentaplegic (R-Smad) proteins are phosphorylated and accumulate in the nucleus. The accumulated Smads subsequently inhibit the transcription of several genes by interacting with DNA binding proteins and transcriptional regulators (15-18). Another study showed that the TGF-β signaling may be inhibited by SnoN and Ski, Smad pathway inhibitors, during the proliferative phase of liver regeneration (19). Increased expression of TGF-β and activation of Smad2 have also been found in a T cell-mediated hepatitis model following PH, and were associated with impaired hepatocellular proliferation (20). The activation of Smad2/3, which can be inhibited by Smad7, is required for TGF-β to have an inhibitory role in liver regeneration (21). Only activated TGF-β has the ability to inhibit cell proliferation, as reported by Schrum *et al,* who observed that activated TGF-β, but not latent TGF-β, affected liver regeneration (22). A previous study found that the activation of TGF-β required the involvement of thrombospondin-1 (TSP-1). Significantly reduced TGF-β/Smad signaling and accelerated hepatocyte proliferation were observed in mice with TSP-1 deficiency following PH (23).

With the exception of EGF, other proliferation-associated factors and signaling pathways can be affected by TGF-β. TGF-β can inhibit the secretion of human hepatocyte growth factor (HGF), which is known to be a complete mitogen in the process of liver regeneration (24). DNA synthesis induced by TNF can also be inhibited by TGF-β in cultured hepatocytes (25). The overexpression of TGF-β1 was shown to inhibit the protein level of cdc25A, a cyclin-dependent kinase-activating tyrosine phosphatase, by enhancing the binding of histone deacetylase 1 to p130 in Alb-TGF-β1 transgenic mice following PH (26). TGF-β1 was found to inhibit DNA synthesis of the G1 stage in cultured hepatocytes treated with HGF and heparin-binding epidermal growth factor-like growth factor by decreasing the expression of cyclin E without affecting the activity of c-Met, epidermal growth factor receptor (EGFR) or mitogen-activated protein kinase (MAPK), or the expression of cyclin D1 (27). This indicated that TGF-β1 has the ability to restrain the growth factor-induced signals between cyclin D1 and cyclin E (27).

Inducing cell apoptosis is important for TGF-β to exert its anti-proliferative effect in liver regeneration. Studies have indicated that liver cell regeneration and atypical bile duct proliferation are associated with TGF-β in fulminant hepatitis (28). The overexpression of activated TGF-β by adenovirus vectors enhanced the apoptosis of hepatocytes, resulting in the death of rats following two-thirds PH (22). Enhanced apoptosis was also found to be accompanied by the overexpression of TGF-β1 in tetracycline-controlled TGF-β1 mice (29), whereas pro-apoptotic signals were suppressed by insulin-like growth factor binding protein-1 (30). In experiments performed by Samson *et al*, the overexpression of TGF-β1 resulted in increased expression of c-Jun, a potential pro-apoptotic transcription factor, which indicated that TGF-β1 induced hepatocyte apoptosis through a c-Jun-independent mechanism (31). The expression of TGF-β1 increases the generation of ROS, an essential mediator of apoptosis, and induces the apoptosis of hepatocytes in liver regeneration, which is associated with promoting the generation of ROS from mitochondria and inducing cytochrome P450 1A1 (32).

TGF-β is also the target of other proliferation inhibiting factors in liver regeneration. Cation-independent mannose 6-phosphate receptor (CIMPR), an indirect negative regulator of hepatocyte growth, which has progressively overexpressed in liver regeneration 8 h after PH, mediates the activation of latent pro-TGF-β (33). A schematic diagram of the TGF-β signaling pathway is shown in Fig. 1.

Activin, another member of the TGF-β superfamily, which is structurally related to TGF-β, also has an inhibitory effect on hepatocyte proliferation and liver regeneration. Activin A, with a similar ability to that of TGF-β, inhibits EGF-induced DNA synthesis in an autocrine manner without competing with TGF-β (34). A study by Schwal *et al* indicated that activin induced the apoptosis of hepatocytes in a different manner from that of TGF-β (35). The administration of follistatin, an activin-binding protein that can inhibit the activity of activin, enhances liver regeneration following PH (35-37). The inhibitory effect of activin may also be inhibited by increased expression of SnoN and Ski, which are Smad pathway inhibitors, in early liver regeneration (19). The impairment of activin type I receptors has also been shown to increase DNA synthesis in hepatocytes, providing further evidence of the anti-proliferative function of activin (38).

*IL-1*. IL-1 is another significant negative regulator of cell proliferation in the process of liver regeneration. In 1988, Takahashi *et al* first reported that IL-1 may be involved in the suppression of liver regeneration on examining syngeneic spleen cells treated with poly I:C in a mouse PH model (39). Another study by Nakamura *et al* showed that IL-1β markedly inhibited DNA synthesis induced by insulin and EGF in cultured rat hepatocytes (40). Similar inhibition of cultured hepatocytes has also been found in other studies (41-43), and enhanced liver regeneration by FK506 can be inhibited by IL-1 (44). The increased expression of IL-1 impairs regeneration via an indirect mechanism in liver injury induced by endotoxin following hepatectomy (45). The mRNA expression of IL-1α in the whole rat liver was found to be downregulated at 10 h, and upregulated 24 and 48 h following PH, suggesting an association between the expression of IL-1 and liver regeneration (46). Further experiments have revealed that sensitivity of hepatocytes isolated from the liver 24 h following PH was increased to the inhibition of IL-1, and the administration of exogenous IL-1β suppressed DNA synthesis at 0 and 12 h post-PH (46). Long-term examination of the expression of IL-1 in the liver in liver regeneration showed that the mRNA levels of IL-1 increased slowly and marginally following 30 and 80% hepatectomy (47). The increased expression of IL-1β was has also been observed in a shrinking liver lobe of a rat portal vein ligation model, indicating that IL-1β was involved in the process of cellular atrophy (48,49). The expression of IL-1β was also increased in the regenerating impaired liver caused by deficiency in the expression of peroxisome proliferator-activated receptor α (50). The suppression of IL-1β by FR167653, a selective inhibitor, was shown to promote liver regeneration in rat models of classic PH (70%) and extended hepatectomy (90%) (51,52). Hepatocyte proliferation was also found to be enhanced by recombinant human interleukin 1 receptor antagonist in a mouse model of acute liver injury induced by $CCl_4$ (53).

IL-1 is secreted by nonparenchymal cells (NPCs) in the regenerating liver. Goss *et al* found that IL-1 is produced by KCs and regulated by prostaglandin E2 (PGE2) in the rat liver following PH (54,55). Heparin and PGE1 also suppress the expression of IL-1 in KCs following PH (45). Increased mRNA levels of IL-1α and β, which are associated with KCs and the spleen, were reported in liver NPCs between 30 min and 1 h following PH (56). Medium conditioned by nonparenchymal cells isolated from the regenerating liver inhibit DNA synthesis in primary rat hepatocytes induced by HGF, EGF and TGF-α. This suppression is inhibited by either IL-1 receptor antagonist or by IL-1 α and β antibodies (46).

Although studies on the association between IL-1 and liver regeneration have been performed, the inhibitory mechanism of IL-1 on liver regeneration remains to be fully elucidated. Several studies have shown that IL-1 receptor antagonist (IL-1Ra), a competitive inhibitor of IL-1α and IL-1β, and anti-inflammatory protein, inhibited the inhibition of IL-1 and facilitated liver regeneration, suggesting that IL-1R was required for the inhibitory action of IL-1 (53,57-59). IL-1 was found to contribute to the impairment of liver regeneration by reducing HGF and promoting TGF-β release in reduced-size orthotopic liver transplantation models (59). A previous study showed that IL-1β inhibits the fibroblast growth factor (FGF)19 signaling pathway, which regulates cell growth and metabolism of hepatocytes in liver regeneration (60). IL-1β also inhibits the expression of β-Klotho, a co-receptor of FGF receptor 4 (FGFR4), with involvement of the c-Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB pathways. The activation of extracellular signal-regulated kinase (Erk)1/2 and hepatocyte proliferation induced by FGF19 is also suppressed by IL-1β.

*IL-6*. IL-6 is a multifunctional cytokine, which has been investigated extensively. It is a known classical triggering signal of liver regeneration following PH. The essential role of IL-6 and its mechanisms in the process of liver regeneration have been confirmed (61). IL-6 has been shown to act as a positive regulator of liver regeneration in the majority of studies, whereas it has also been shown that IL-6 impairs liver regeneration, which conflicts with the mainstream findings. IL-6 inhibits mature hepatocyte proliferation and DNA synthesis stimulated by TNF and EGF in primary culture (25,42). Beyer and Theologides showed that IL-6 suppressed the DNA synthesis of hepatocytes at 3 days *in vitro* (43). The inhibitory effect of IL-6 in hepatocyte proliferation may be associated with induction of the expression of p21 *in vitro* (62).

Upregulated levels of IL-6 have been observed in a number of abnormal liver regeneration models. Increased blood levels of IL-6, which induce the expression of protein inhibitor of activated Stat-3 and the suppressor of cytokine signaling (SOCS)-1, were found to be associated with impairment of liver regeneration in a model of hepatic failure (63). Hyperstimulation with IL-6 also suppressed hepatocyte proliferation by inducing the expression of p21 in transgenic mice overexpressing the human soluble IL-6 receptor/gp80 following PH (64). The overexpression of STAT3, a downstream molecule of IL-6, was found to impair hepatocyte proliferation in fatty liver following PH (65). In addition, the suppression of hepatocyte proliferation was accompanied by

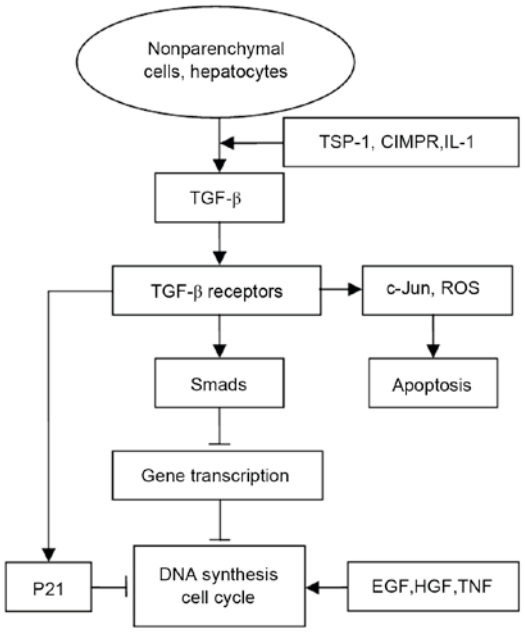


Figure 1. Schematic diagram of TGF-β signaling pathways. TGF-β, transforming growth factor-β; Smads, small mothers against decapentaplegic; TSP-1, thrombospondin-1; CIMPR, cation-independent mannose 6-phosphate receptor; IL-1, interleukin-1; ROS, reactive oxygen speces; EGF, epidermal growth factor; HGF, hepatocyte growth factor; TNF, tumor necrosis factor.

increased levels of IL-6 in IL-1Ra-knockout mice following PH (57). The impairment of liver regeneration by hepatitis B virus X protein (HBx) via upregulating the expression of IL-6 was observed in an HBx-overexpressed transgenic mouse model (66). In addition to the data obtained in animal models, elevated levels of IL-6 have also been found in patients with chronic liver disease (67-70).

Although the mechanisms of IL-6 in liver regeneration are controversial, the majority of associated studies support the positive role of IL-6, with IL-6 affecting hepatocyte proliferation in a dose- and time-dependent manner (71,72). The studies mentioned above also indicated that the inhibitory effects of IL-6 in liver regeneration, particulary on a background of liver disease, may be associated with its hyperstimulation of liver cells.

*SOCS*. SOCS is a family of proteins, which negatively regulate various cytokine signals (2). SOCS inhibits the activity of STAT3, a downstream signal of cytokines and growth factors in hepatic regeneration. SOCS3, a feedback inhibitor of the IL6/JAK/STAT3 pathway, is an important member of the SOCS family, which has received the most attention in investigations of liver regeneration. SOCS3 is transiently upregulated in the livers of mice following PH and is involved in the inactivation of STAT3 signaling (73-75). SOCS3 can inhibit the phosphorylation of gp130, a component of the IL-6 receptor, JAKs and STATs (76). SOCS3 hepatocyte-specific-knockout mice showed increased capability of cell proliferation and prolonged STAT3 phosphorylation following PH, with enhanced activation of ERK1/2 also observed (77). SOCS3 can also negatively control the proliferative responses mediated by HGF and EGF (78). The overexpression of SOCS3 suppresses IL-22 signaling, another cytokine positively correlated with liver regeneration, via eliminating the IL-22-induced activation of STAT (79). The expression of SOCS3 is primarily induced by IL-6 and is also upregulated by other factors, including TNFα, IL-1, IL-22 and ZIP14, a zinc transporter (73,79-81).

SOCS1, another member of the SOCS family, has a similar but weaker function to that of SOCS3 (74,78). Unlike SOCS3, SOCS1 does not affect the growth signaling mediated by EGFR (78). A previous study indicated that SOCS1 negatively regulated the hepatocyte proliferation induced by HGF by inhibiting c-Met signaling (82).

*IFN-γ*. IFN-γ can downregulate hepatocyte proliferation by activating STAT1 and its downstream genes, including RF-1, p21 and SOCS1, in liver regeneration (83). The inhibitory effect of invariant natural killer T (iNKT) cells, a major lymphocyte in the liver, was also associated with IFN-γ (84). iNKT cells affect liver regeneration to a lesser degree. The deficiency of iNKT cells (CD1d and Jα281 cells) does not alter mouse liver regeneration following PH. However, treatment with α-galactosylceramide, an inducer of iNKT cell activation, impairs hepatocyte proliferation via upregulating the expression of IFN-γ in wild-type mice. Experiments also indicated that IL-4 is the primary mediator in the activation of iNKT cells and induction of IFN-γ (84).

*Other cytokines*. There are other cytokines, which negatively regulate liver regeneration. TNF-α, another pro-proliferation factor in liver regeneration, also has the ability to induce the apoptosis of adenovirus-infected hepatocytes in a coupled TGF-α-IL-1α/β-IL-1ra autocrine cascade manner (85). The administration of α2b-INF inhibits DNA biosynthesis and liver mass restitution by affecting the expression of cell cycle-associated genes, includin c-myc, p53 and c-erbB-2, in rat PH models (86). Further experiments have indicated that the suppression of hepatocyte proliferation depends on the time at which α2b-IFN is administered (87), and the proliferation of hepatocytes was found to be inhibited by granulocyte colony-stimulating factor via upregulating the expression of IL-1β in a liver injury model induced with dimethylnitrosamine (88). Mice with single deficiency of myeloid differentiation factor 88 (MyD88), an adaptor protein for the majority of Toll-like receptors, presented with enhanced hepatocyte proliferation at 32 and 36 h post-PH (89). The decreased activation of STAT3 and induction of SOCS3 have also been detected in MyD88-null mice following PH (89). These results suggest that MyD88 has an antiproliferative effect in liver regeneration by affecting IL-6 signaling pathways. A summary of the cytokines involved in inhibiting liver regeneration is presented in Table I.

### 3. Oncogene-associated pathways

*P53*. The role of p53, a negative regulator of growth and tumor suppressor genes, is complex in the process of liver regeneration and conclusions from different studies are contradictory. The expression of p53 is upregulated, corresponding to the G1/S transition, in the early stage of liver regeneration following PH (9,90). It has been shown that the overexpression of p53 by a transducing adenoviral vector encoding wild-type p53, did not affect the process of liver regeneration (91), however,

Table I. Cytokines involved in inhibition of liver regeneration.

| Cytokine | Source | Upstream | Downstream |
|---|---|---|---|
| TGF-β | NPCs (HSCs, KCs and platelets), hepatocytes | TSP-1, CIMPR, IL-1 | TGF-β receptors, Smads (inhibit gene transcription) c-Jun, ROS (promote apoptosis) |
| IL-1 | KCs | Heparin, PGE1, PGE2, G-CSF | IL-1 receptor, TGF-β, FGF19, β-Klotho, JNK, NF-κB, Erk1/2 |
| IFN-γ | iNKT cells | IL-4 | STAT1 and downstream genes (RF-1, p21 and SOCS1) |
| α2b-IFN | Ectogenesis | | Cell cycle-related genes (c-myc, p53 and c-erbB-2) |
| G-CSF | Ectogenesis | IL-1 | |
| MyD88 | Hepatocytes | | STAT3 (inhibition), SOCS3 (induction) |

TGF-β, transforming growth factor-β; IL-1, interleukin-1; IFN, interferon; G-CSF, granulocyte colony-stimulating factor; MyD88, myeloid differentiation factor 88; TSP-1, thrombospondin-1; CIMPR, cation-independent mannose 6-phosphate receptor; ROS, reactive oxygen species; FGF19, fibroblast growth factor 19; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; Erk, extracellular signal-regulated kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling.

other studies showed that p53 may positively regulate hepatocyte proliferation in rats. The suppression of the expression of p53 results in the reduction of DNA replication in hepatocytes *in vitro* (92). A study by Inoue *et al* showed that the levels of p53 increased in cultured hepatocytes induced with HGF, and inhibiting the expression of p53 by an antisense oligonucleotide resulted in a decrease in the levels of TGF-α and of DNA synthesis (93). It was also found that upregulation of the expression of p53 was synchronous with HGF followed by an increase of TGF-α in a rat PH model, suggesting that p53 is positively correlated with liver regeneration (93).

However, other findings have supported that p53 negatively controls liver regeneration. Previous studies have provided indirect evidence of the anti-proliferation functions of p53. Suppressing p53 with antisense oligonucleotides enabled hepatocytes to recover from growth arrest induced by ultraviolet (94). Increased expression of p53, accompanied by a downregulation in the levels of proliferating cell nuclear antigen (PCNA), was observed in rats treated with a metallothionein antisense oligonucleotide, suggesting an inverse correlation between p53 and liver regeneration (95). The detailed role of p53 in liver regeneration was examined by Arora and Iversen (96). Increased weight gain of remnant-regenerating liver, CYP isoform activities and expression of PCNA were observed in rat PH models, in which the expression of p53 was inhibited with an antisense oligonucleotide, whereas the $G_1$ cell populations and levels of P21 were also reduced in the p53-inhibited rats, indicating that p53 controls hepatocyte proliferation in a cell cycle checkpoint manner (96). Furthermore, loss of the expression of p53 resulted in early entry into the cell cycle and prolonged the proliferation of hepatocytes in p53 (-/-) mice following PH, and the inhibitory mechanism of p53 was correlated with the expression of mitotic genes, including Forkhead box M1, Aurka, Large tumor suppressor kinase 2, Polo like kinase (Plk)2 and Plk4 (97). Abrogation of the p53 pathways also facilitates liver regeneration in Wild-type p53-induced phosphatase 1-deficient mice following PH (98).

P53 can also suppress cell proliferation in regeneration of damaged liver on a background of chronic disease or drug treatment. An increased expression of p53 followed by the induction of p21 was associated with the inhibition of hepatocyte proliferation in PH models treated with 2-acetaminofluorene (99). Impaired liver regeneration and increased expression of p21 following partial PH was observed in mice lacking c-Jun, a key regulator of hepatocyte proliferation, whereas the activation of p53 eliminates the effect, indicating that p53 is a proliferation inhibitor in liver regeneration (100). The activation of a p53-dependent checkpoint correlates with premitotic arrest and abnormal cell death in transgenic mice overexpressing human Aurora-A following PH (101). The expression levels of p53 and p21 are also upregulated, coinciding with cell cycle arrest and apoptosis in hepatocytes of liver injury models induced by acetaminophen (102-104).

*P21.* P21, the expression product of the ras gene, also termed wide-type 53-activated factor 1 or cyclin-dependent kinase interacting protein 1, has been identified as a cyclin-dependent kinase inhibitor and downstream gene of p53 (105-107). The negative control role of p21 has been confirmed in various studies. The expression of p21 increased maximally following peak hepatocyte DNA synthesis and cell division, later than that of p53, in rat and mouse PH models, indicating its association with the proliferation of liver cells (90,108). The upregulation in levels of p21 have been examined not only hepatocytes, but also nonparenchymal cells, in the process of liver regeneration (109). In addition to the data mentioned above, overexpressed p21 also coincides with the arrestment of liver regenerating in several disease and drug toxicity models (65,110-115).

Although p21 is a classic downstream signal of p53, the expression of p21 is induced by other factors in addition to p53 in liver regeneration. Increased mRNA levels of p21 induced by p53 were identified and involved in inhibiting hepatocyte proliferation in 2-acetaminofluorene-treated rats (120). Upregulated levels of p53 and p21 were also correlated with impaired liver regeneration in acute liver injury models induced by acetaminophen, and inhibition of the p53/p21 pathway promoted hepatocyte proliferation (102,121).

Table II. Oncogenes involved in inhibition of liver regeneration.

| Oncogene | Upstream | Downstream | Function |
|---|---|---|---|
| P53 | | P21 (upregulating) | Cell cycle checkpoint |
| P21 | P53, TGF-β, activin A, C/EBPα | Cell cycle-related protein (downregulating) | Cell cycle checkpoint |
| Bcl-2 | | p107, cyclin E (downregulating) | Negatively modulates cell cycle |
| PUMA | | Bax, macrophage inflammatory protein-2 | Another member of the Bcl-2 family |
| BI-1 | | Cell cycle-related protein (downregulating) | Negatively modulates cell cycle |
| P27 | | CDK2 (downregulating) | Negatively modulates cell cycle |
| Fas | | - | Induces apoptosis |
| NDRG2 | | P53/p21 | Negatively modulates cell cycle |

TGF-β, transforming growth factor-β; C/EBPα, CCAAT/enhancer-binding protein-α Bcl-2, B-cell lymphoma 2; PUMA, P53 upregulated modulator of apoptosis; BI-1, BAX, Bcl-2-associated X protein; BI-1, BAX inhibitor 1; CDK2, cyclin-dependent kinase 2; NDRG2, N-Myc downstream-regulated gene 2.

However, no difference in the gene expression of p21 was found between p53-deficient and wild-type mice following PH, although the post-transcriptional regulation may be associated with p53 (116). Furthermore, treatment with TGF-β and activin A upregulated the expression of p21 in cultured primary hepatocytes (116). These data suggested that the induction of p21 is controlled in a p53-dependent and independent manner. Similar expression and localization of p21 was also found in wild-type and p53-null mice treated with carbon tetrachloride, and the increased levels of p21 might be correlated with CCAAT/enhancer-binding protein (C/EBP) (117). Other experiments have shown that levels of p21 were regulated by C/EBPα (122). Increased levels of p21 were observed in c-Jun-knockout mice following PH, and inactivation of the p53/p21 signaling pathway abrogated the suppression of liver regeneration, suggesting that the expression of p21 may be regulated by c-Jun (100).

*B-cell lymphoma-2 (Bcl-2).* Important in the proliferation of tumor cells, Bcl-2 also inhibits hepatocyte proliferation in liver regeneration. Bcl-2 transgenic mice show delayed hepatocyte DNA synthesis following PH, and a similar result is observed in cultured liver cells. In addition, the overexpression of Bcl-2 is followed by decreased expression levels of p107 and cyclin E (123). These results indicate that Bcl-2 may be a negative cell cycle modulator in liver regeneration.

P53 upregulated modulator of apoptosis (PUMA), another member of the Bcl-2 family, is also negatively correlated with liver regeneration. PUMA is downregulated, accompanied by increased apoptosis and decreased proliferation, in the early phase post-PH (124). Bcl-2-associated X protein (Bax) inhibitor-1 (BI-1), which has a similar function to that of Bcl-2 family proteins, also regulates liver cell proliferation *in vivo*. Enhanced liver regeneration, accompanied by the increased expression of cell cycle regulators, including cyclin D1, cyclin D3, Cdk2 and Cdk4, have been observed in BI-1-deficient mice following PH (125).

*Other oncogene-associated pathways.* Several studies have shown that additional oncogenes can inhibit liver regeneration. P27 is also a tumor suppressor gene and cyclin-dependent kinase inhibitor. P27 was shown to be minimally expressed in the mouse liver following PH in a time-dependent manner, and p27 decreased the activity of CDK2 prior to and following peak DNA synthesis, suggesting its negative role in liver regeneration (108).

Fas, also known as APO-1 or CD95, and Fas ligand (FasL) are essential in cell apoptosis and regulate the proliferation of tumor cells (126). The expression of Fas decreased significantly immediately in the rat liver and had recovered gradually to a normal level of quiescent hepatocytes 14 days following PH, suggesting that the Fas/ FasL system was also negatively correlated with liver regeneration (127).

N-Myc downstream-regulated gene 2 (NDRG2), is also a known tumor suppressor gene and its expression is reduced or undetectable in various types of tumor (128). The significant downregulation of NDRG2 was examined in regenerating livers, and the overexpression of NDRG2 resulted in a cell cycle arrest via upregulating the p53/p21 pathway and inhibiting cyclin E-Cdk2 activity (129). These data indicate that NDRG2 has a potential negative effect in liver regeneration. A summary of the oncogenes involved in inhibiting liver regeneration is presented in Table II.

**4. Gene expression regulation-associated pathways**

*Transcription factor-associated pathways.* Transcription factors important roles in liver regeneration, however, the majority of them positively regulate liver regeneration. Other transcription factors, which may have inhibitory roles in liver regeneration, are less well understood.

C/EBPα, a member of the CCAAT enhancer-binding protein family, is expressed abundantly in the normal liver. However, the expression of C/EBPα was found to decrease markedly in proliferating cells following PH and in cultured hepatocytes stimulated with EGF and insulin, suggesting an inverse correlation between C/EBPα and liver regeneration (130). Positive correlation between p21 and C/EBPα has been observed in C/EBPα-knockout mice. C/EBPα did not affect the mRNA levels of p21 but inhibited the proteolytic

Table III. Protein modification-associated pathways in liver regeneration inhibition.

| Name | Downstream | Function |
|---|---|---|
| SOCS | STAT3 | Downregulating STAT3 phosphorylation |
| PTP1B | JNK1/2, STAT3, EGFR, HGFR | Dephosphorylating tyrosine kinases |
| Hippo pathway | YAP | Inhibiting proliferation-associated transcription factors |
| PPM1A | Cyp3a11 | |
| Mig-6 | Protein kinase B, EGFR | Modulating phosphorylation of EGFR |

SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; PTP1B, protein tyrosine phosphatase 1B; JNK, c-Jun N-terminal kinase; EGFR, epidermal growth factor receptor; HGFR, hepatocyte growth factor receptor; YAP, Yes-associated protein; PPM1A, Mg(2+)/Mn(2+)-dependent phosphatase 1A; Mig-6, mitogen-inducible gene-6.

degradation of p21, indicating that C/EBPα inhibited hepatocyte proliferation via controlling the expression of p21 in a post-transcriptional manner (122). In aged animal PH models, C/EBPα also suppressed the expression of E2F via forming the C/EBPα-Rb-E2F4 complex, which can bind to E2F-dependent promoters, inhibiting the expression of E2F and inhibiting cell proliferation (131).

Forkhead box (Fox)O1, a transcription factor regulated by Akt, may also be an anti-proliferative factor in liver regeneration. Deleting Akt1 and Akt2 was shown to result in impairment of liver regeneration in mice following PH, whereas liver-specific deletion of FoxO1 reversed this effect, indicating that FoxO1 has a negative effect on liver regeneration (132).

## 5. MicroRNAs (miRNAs) and the inhibition of liver regeneration

MicroRNAs (miRNAs) have an effect on the post-transcriptional control of gene expression and their association with liver regeneration has attracted increasing interest. A number of miRNAs have been identified as liver regeneration inhibitors, including miR-26a, miR-33, miR-34a, miR-127, miR-150 and miR-378 (133).

## 6. Protein modification-associated pathways

Previous studies have shown that certain anti-proliferation inhibitors are involved in liver regeneration via regulating the protein modification of downstream molecules, including phosphorylation. Of note, SOCS, the natural terminators of cytokine signaling, are involved via regulating the phosphorylation of downstream molecules (76). Tyrosine phosphorylation-mediated signaling, including the EGF and HGF pathways, is required in liver regeneration, whereas protein tyrosine phosphatase 1B (PTP1B), a key regulator of metabolism and cell growth, is involved in the dephosphorylating process of the tyrosine kinase superfamily. PTP1B-deficient mice present with enhanced cell proliferation and increased phosphorylation of JNK1/2 and STAT3, the downstream molecules of TNF-α and IL-6, which trigger liver regeneration following PH (134). Similar phosphorylation was observed in wild-type cells following the silencing of PTP1B, whereas the overexpression of PTP1B led to the opposite result. Accumulated tyrosine phosphorylation of EGFR and hepatocyte growth factor receptor (HGFR), followed by increased activation of Akt and ERK, were also examined in PTP1B-/- mice following PH. These data indicate that PTP1B negatively regulates liver regeneration via dephosphorylating key molecules of the signaling pathways, which promotes cell proliferation.

The Hippo pathway is a novel pathway, which is associated with organ size control and tumorigenesis (135). The Hippo pathway consists of several core kinases, including the mammalian Ste20-like kinases (Mst1/2) and Salvador 1, and activates large tumor suppressor (Lats1/2), exerting effects via a series of phosphorylating processes of these kinases. The final effect of the Hippo pathway is to phosphorylate and inhibit the activity of Yes-associated protein (YAP), a transcription co-activator, and the transcriptional co-activator with PDZ-binding motif, which regulates different proliferation-associated transcription factors. Increased activation of YAP has been found in the early stage of rat liver regeneration following PH, whereas the activation of Mst1/2 and Lats1/2 were downregulated at this time (136). The levels of YAP and Mst1/2 kinase returned to their normal levels, as in quiescent liver cells, when the liver size was almost restored, suggesting a negative role of the Hippo pathway in liver regeneration.

Mg(2+)/Mn(2+)-dependent phosphatase 1A (PPM1A) is another phosphatase identified in a previous study, which has inhibitory potential in liver regeneration. The expression of PPM1A was decreased, which was positively correlated with the expression of Cyp3a11 in the early stage of liver regeneration in mice (137). Furthermore, inhibition of the expression of PPM1A leads to the enhanced proliferation of HepG2 cells. The detailed mechanism of PPM1A in the control of liver regeneration requires further investigation in the future.

Mitogen-inducible gene-6 (mig-6), an adaptor protein associated with the modulation of receptor tyrosine kinases, including EGFR, is also known as receptor-associated late transducer, gene33 and Errfi1 (138,139). In mig-6-knockout mice, accelerated hepatocyte proliferation is observed in the early stage of liver regeneration following PH. Downregulating the expression of mig-6 also enhances EGFR signaling via the protein kinase B pathway in mouse PH models (140), suggesting that mig-6 is a negative regulator in liver regeneration. A summary of the protein modification-associated pathways involved in the inhibiton of liver regeneration is shown in Table III.

### 7. Metabolism-associated pathways

Metabolism is an important function of the liver and also affects the process of liver regeneration. Several metabolic factors have been reported to be associated with the impairment of liver regeneration. For example, very low-density lipoprotein has been shown to suppress hepatocyte proliferation in *in vitro* and *in vivo* experiments (141). The overadministration of glucose has also been shown to arrest DNA synthesis and cell cycle in liver injury models, indicating that glucose loading depresses liver regeneration (142).

S-adenosylmethionine (SAMe), the primary methyl donor in the liver, is important in the metabolism of cells and regulates cell proliferation. Adenosine monophosphate-activated protein kinase (AMPK), the principal energy sensor in cells, can be inhibited by SAMe. A study by Vazquez-Chantada *et al* showed that hepatocyte proliferation was inhibited by SAMe in mouse liver regeneration (143). SAMe inhibited the HGF-mediated phosphorylation of AMPK, which upregulated cytoplasmic HuR and certain cell cycle proteins, including cyclin D1 and A2 in liver cells. Furthermore, phosphorylation of the LKB1/AMPK/endothelial nitric oxide synthase cascade mediated by HGF was suppressed in mouse PH models pretreated with SAMe. Evidence of the inhibitory function of SAMe on AMPK and liver regeneration was also obtained in another study (144).

Sirtuin 1 (SIRT1) is an important metabolic modulator of gluconeogenesis, fat, protein and bile acid (BA), and is also associated with liver regeneration. Impaired hepatocyte proliferation accompanied by BA accumulation and damaged farnesoid X receptor (FXR) activity was observed in transgenic mice overexpressing SIRT1 following PH (145). The overexpression of SIRT1 resulted in the persistent deacetylation of FXR, which was inversed by 24-norursodeoxycholic acid, followed by decreased expression of FXR-target genes, including small heterodimer partner and bile salt export pump. These data indicate that SIRT1 may negatively regulate liver regeneration by controlling the metabolism of BA.

### 8. Hormone-associated pathways

The association between hormones and liver regeneration has been investigated in previous studies (146). Norepinephrine upregulates the level of TGF-β via the α1-adrenergic receptor (α1-AR) in cultured rat hepatocytes (147). The levels of adrenal hormones are negatively correlated with DNA synthesis and cell cycle arrest, in a circadian manner, during liver regeneration in hepatectomized animals (148). The expression and function of α1-AR are decreased, in contrast to the increased level of β2-AR, in hepatocytes following PH. Of note, treatment with isoproterenol, a β-AR agonist, inhibits 3H-thymidine incorporation of hepatocytes isolated from PH models via upregulating the activation of stress-activated protein kinase and inhibiting p42 MAP kinase. This was inhibited by propranolol, a β-AR antagonist (149).

Hydrocortisone treatment results in the persistent suppression of hepatocyte proliferation and increased expression of P21 in PH models (150). Pretreatment with dexamethasone also inhibits the proliferation of hepatocytes, accompanied by reduced expression levels of TNF and IL-6 (151). This suggests that glucocorticoid hormone has an inhibitory effect on liver regeneration. Short-term administration of ethinyl estradiol, a potent promoter of hepatocarcinogenesis in female rats, leads to an initial, transient increase in hepatocyte proliferation. However, long-term treatment with ethinyl estradiol results in the inhibition of liver regeneration, without affecting DNA synthesis, following PH (152). Somatostatin also inhibits DNA synthesis induced by HGF and EGF via a cAMP-independent mechanism, indicating that it may be a potent inhibitor of liver regeneration (153).

### 9. Pathological factors and liver regeneration

Pathological alterations caused by various factors, including ethanol, drugs and hepacivirus, on liver regeneration is generally accepted. The abnormal expression of cytokines and proliferative inhibitors, induced by acute or chronic pathological changes, is associated with the impairment of liver regeneration. Their association was discussed above.

Hepatitis, cirrhosis and hepatic fibrosis, the most common pathological changes in the liver, negatively affect liver regeneration. T cell-mediated hepatitis leads to reduced liver regeneration via altered expression levels of certain cytokines and associated signaling molecules, for example the upregulated expression of p21, Smad2, TGF-β and IFN-γ, and downregulated expression of cyclin D and activation of Stat3 (20). In addition to the involvement of cytokines, other factors are associated with impaired regeneration in liver disease models. For example, hypoxia in hepatocytes caused by cirrhosis suppresses the hepatocyte proliferation induced by HGF and c-Met *in vivo* (154). The activation of HSCs, the primary cause of hepatic fibrosis, also inhibits liver regeneration and can be attenuated by STI-571 (155).

The consumption of ethanol, which leads to ethanol-induced liver injury, is another common harmful factor affecting liver regeneration. Long-term ethanol intake inhibits hepatic DNA synthesis following PH, which is associated with a change in hepatocyte sensitivity to TNF (156). Acute and chronic ethanol consumption can inhibit liver regeneration by different mechanisms. The suppression of rat liver regeneration by acute ethanol is accompanied by upregulated levels of p53, prohibitin and TGFβ-1 (157). Prolonging of the activation of p42/44 MAPK and decreased expression of γ-aminobutyric acid transport protein are also involved in the damaged proliferation of hepatocytes (158,159). By contrast, prothymosin-α, p38 MAPK and p21 are associated with impaired liver regeneration following chronic ethanol administration (158,160,161).

Fatty liver also exhibits impaired regenerative capacity. The over-phosphorylation of Stat-3, inhibition of Jun N-terminal kinase, and decreased expression of NF-κB and cyclinD1 were found to be associated with the inhibition of proliferation in mouse nonalcoholic fatty liver disease models following PH (162). Furthermore, enhanced activation of STAT3 was found to be positively correlated with the levels of p21 in the regenerating process of fatty liver models (65).

The administration of drugs is another common factor in liver injury and the inhibition of liver regeneration. For example, amiloride, a weak diuretic and inhibitor of $Na^+/H^+$ exchange, has the ability to suppress DNA synthesis of proliferating hepatocytes *in vitro* and *in vivo* (163,164). The amiloride

analog, 5-(N, N-hexamethylene)-amiloride, another inhibitor of $Na^+/H^+$ exchange, not only inhibits DNA synthesis, but also leads to apoptosis of hepatocytes in the rat liver following PH. Induction of the p53/p21 pathway is associated with the inhibitory function of 5-(N, N-hexamethylene)-amiloride (164).

The toxicity caused by intake of certain microelements can also damage liver regeneration. For example, selenium, a non-metal element, was shown to arrest DNA synthesis and cell cycle in rat PH models, which may be associated with the reduction of glutathione (165). Cadmium, a heavy metal element, is also harmful to liver regeneration involving inhibition of the enzymatic activity of thymidine kinase (166).

## 10. Other inhibitors of liver regeneration

There are certain proliferation inhibitors, which cannot be classified into the categories mentioned above. Regucalcin, a $Ca^{2+}$-binding protein, was found to be downregulated in the rat liver at 1-3 days post-PH. Inhibition of nuclear DNA synthesis was found to be caused by regucalcin in hepatocytes isolated from PH rats, and $Ca^{2+}$ treatment (1.0-25 $\mu$M), which led to a similar result, indicated the negative effect for regucalcin in liver regeneration (167).

Peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear receptor, which induces cell-cycle arrest and apoptosis in tumor cells, appears to have a negative correlation with liver regeneration (168). The expression of PPAR-γ was found to decrease in the rat liver at 24 h and increase at 48-120 h post-PH, whereas the consumption of pioglitazone, a PPAR-γ agonist, suppressed the proliferation of liver cells.

Vitamin D3 upregulated protein 1 (VDUP1), a regulator of cellular metabolism and endoplasmic reticulum (ER) stress, is also a suppressor of cell proliferation in various types of tumor. A previous study showed that VDUP1 was negatively correlated with liver regeneration (169). Enhanced proliferative responses were observed in the process of liver regeneration in VDUP1-knockout mice. The increased expression of cell-cycle proteins and the activation of proliferative signals appeared earlier in the VDUP1-knockout mice following PH.

Transmembrane and ubiquitin-like domain containing 1 (Tmub1), a novel gene, which is upregulated in the early stage of liver regeneration, was shown to have a negative effect in IL-6-induced hepatocyte proliferation via its interaction with calcium modulating cyclophilin ligand *in vitro* (72). Another study showed that the expression of Tmub1 may be regulated by IL-6 and C/EBPβ (170).

## 11. Conclusion and outlook

Proliferation-inhibiting signals are active in the entire process of liver regeneration, a number of which are induced in the early stage of liver regeneration when the cells are engaged in proliferation. In addition, the expression of other inhibitors are decreased at the same phase, but increased following peak proliferation. They contribute to control of the degree of regeneration and prevent oncogenesis through different mechanisms, with the function of proliferation-inhibiting pathways being as important as proliferation-promoting signals in liver regeneration.

However, knowledge of the proliferation-inhibiting pathways remains less than that of the proliferation-promoting pathways in liver regeneration. The majority of the signaling pathways described above remain to be fully elucidated. A focus is required on the elucidation of proliferation-inhibiting pathways. The identification of additional proliferation inhibitors, together with their upstream and downstream molecules, is required, as is an emphasis on the association between pro-proliferation and anti-proliferation signals.

## Acknowledgements

This study was supported by a grant from the Natural Science Foundation of China (grant no. 81400651).

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