Transforming Growth Factor-β in Liver Cancer Stem Cells and Regeneration

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Cancer stem cells have established mechanisms that contribute to tumor heterogeneity as well as resistance to therapy. Over 40% of hepatocellular carcinomas (HCCs) are considered to be clonal and arise from a stem-like/cancer stem cell. Moreover, HCC is the second leading cause of cancer death worldwide, and an improved understanding of cancer stem cells and targeting these in this cancer are urgently needed. Multiple studies have revealed etiological patterns and multiple genes/pathways signifying initiation and progression of HCC; however, unlike the transforming growth factor β (TGF-β) pathway, loss of p53 and/or activation of β-catenin do not spontaneously drive HCC in animal models. Despite many advances in cancer genetics that include identifying the dominant role of TGF-β signaling in gastrointestinal cancers, we have not reached an integrated view of genetic mutations, copy number changes, driver pathways, and animal models that support effective targeted therapies for these common and lethal cancers. Moreover, pathways involved in stem cell transformation into gastrointestinal cancers remain largely undefined. Identifying the key mechanisms and developing models that reflect the human disease can lead to effective new treatment strategies. In this review, we dissect the evidence obtained from mouse and human liver regeneration, and mouse genetics, to provide insight into the role of TGF-β in regulating the cancer stem cell niche. (*Hepatology Communications* 2017;1:477–493)

Introduction

Cancer stem cells have established mechanisms that contribute to tumor heterogeneity as well as resistance to therapy.[1-4] Yet to date, the switches involved in stem cell transformation in the liver and the definitive role of key pathways involved in liver regeneration and cancer remain partially understood. Multiple studies have revealed etiological patterns and multiple genes/pathways signifying initiation and progression of HCC. These pathways include CTNNB1/WNT-β-catenin, TPp53, ARID1/2s, HGF/c-Met, and vascular endothelial growth factor/angiogenic signaling.[5-12] However, unlike the transforming growth factor β (TGF-β) pathway, loss of p53 and/or activation of β-catenin do not spontaneously drive HCC in animal models.[13-15]

Primary cancer of the liver (HCC) currently remains among the most prevalent and lethal cancers, with ~17% 5-year survival rate (2007–2013).[2,16-18] Drug resistance is one of the causal factors for therapy failure and is associated with the existence of tumor-like stem cells.[15] Yet driving pathways and mechanistic insight into stem cell transformation, leading to targeted therapeutics, remain poorly understood for these cancers. We review insight into how
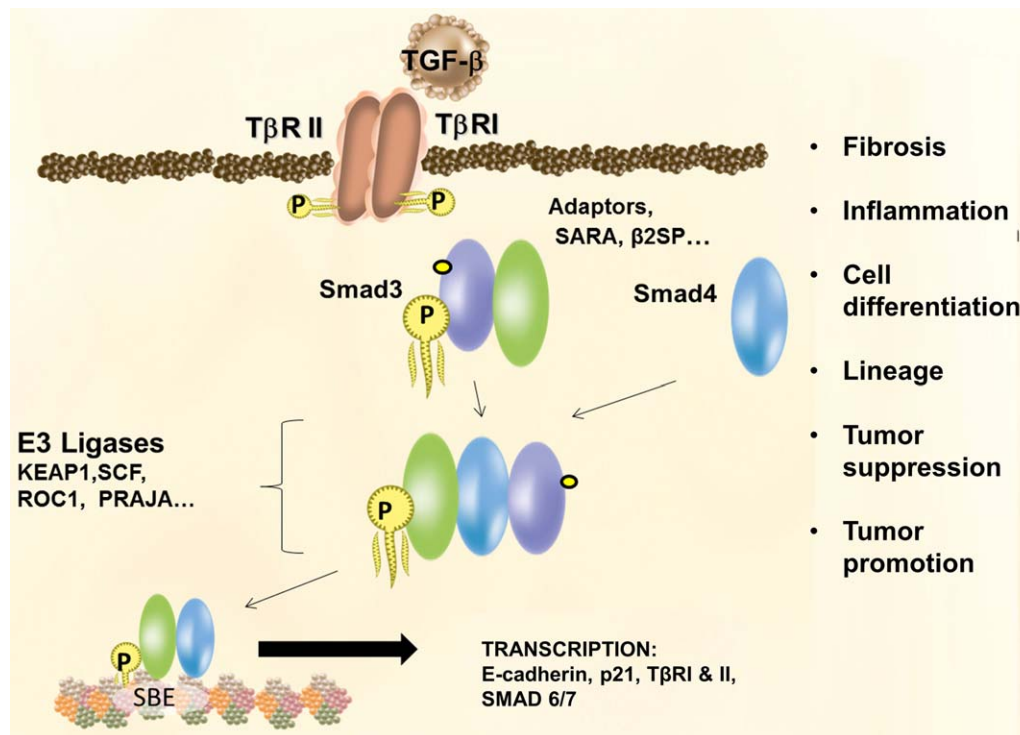
FIG. 1. TGF-β serves as an essential regulator of cell polarity, growth, differentiation, lineage specificity, tumor suppression, and tumor promotion in multiple cell types.

TGF-β drives these cancers and controls the switch from normal stem cells to cancer through mechanistic insight of mouse genetic models.[13,14] TGF-β serves as an essential regulator of cell polarity, growth, differentiation, lineage specificity, tumor suppression, as well as tumor promotion in multiple cell types[13,19] (Figs. 1–3). Yet the significance of dichotomy in function remains unclear for the liver and gastrointestinal system. Defective TGF-β signaling is implicated in multiple cancers due to frequent somatic mutations or deregulation of its components, such as Smad3, Smad4, and TGF-β receptors 1 and 2 (TBRI and TBRII).[15,20,21] Smads are the intracellular mediators of TGF-β signaling,[21-24] and their function is modulated by adaptor proteins such as the Smad anchors and signal transducers, filamin, and microtubules,[25,26] as well as E3 ligases such as SMURFs, Ski, PRAJA, Sno, and others.

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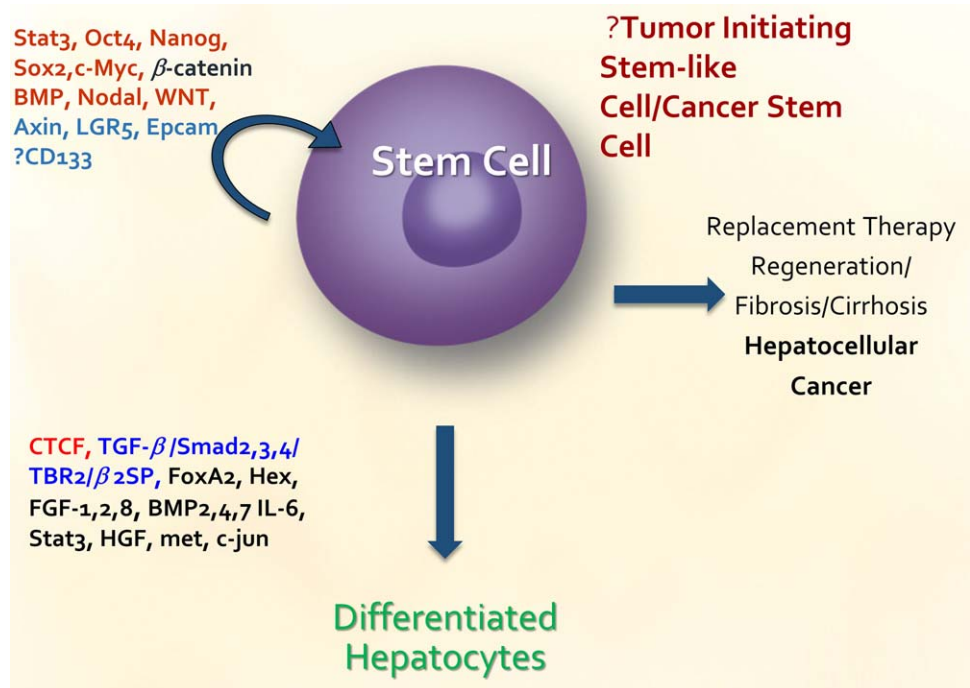
FIG. 2. List of genes that has been identified to be important in liver cancer stem cell or normal stem cell function.

Traditionally, TGF-β has been considered to be mainly prominent during the termination phase of liver regeneration. However, several TGF-β–associated genes have important roles throughout the three phases of liver regeneration and focusing on the temporal fluctuations of these TGF-β–associated partners can provide an insight into their function. This review addresses the role of "core" TGF-β pathway–related genes (based on a literature search) that are grouped into five different categories, including TGF-β "receptors," "ligands," "receptor substrates," "adaptors," and "inhibitory SMADs." These genes are TGF-β1-3, TBRIII, TGF-βRAP1, BMP1-7, BMP9,10, BMP15, BMPR1A, BMPR1B, BMPR2, SMAD1-7, SMAD9, SPTBN1 (β2SP), ACVR1, ACVR1B, ACVR1C, ACVR2A, ACVR2B, ACVRL1, ZFYVE9, INHA, INHBA, INHBB, INHBC, INHBE, GDF1, GDF11, and NODAL. In addition, other molecules, including E3 ligases, are also associated upstream or downstream of the TGF-β pathway and correlate their expression levels with TGF-β pathway activity such as SKI, SMURF1-2, ITCH, ITIH4, SARA, β2SP, ELF1-5, PRAJA1-2, MYC, TERT, RUNX, CTCF, ALDH2, IL-6, STAT3, TWIST1-2, ZEB1, CDK4, TGIF1-2, STRAP, and SNAI1-2.

Liver Stem Cells and TGF-β: Evidence From Mouse Knockout, Lineage Studies, and Human Liver Regeneration

Currently, at least three stem cell populations are known to exist in both mouse[27] and human adult liver[28]: 1) pericentral Axin2^{+} hepatocytes that can regenerate liver in normal homeostasis[29]; 2) periportal cells positive for Lgr5[30]; 3) Prom1^{+} liver stem cells that are located within or adjacent to the Krt19^{+} bile duct epithelium.[31] Human periportal cells also label for octamer 3/4 (Oct3/4), β2 spectrin (β2SP), and TBRII in both human and mouse liver regeneration.[27,28,32] Through previous studies in human liver donor and liver transplant specimens that represent human liver regeneration, as well as fulminant hepatic failure, putative liver progenitor/stem cell expansion has been observed during massive hepatic necrosis with fibrosis as well as submassive hepatic necrosis with hepatocytic lineage. The observation of fibrosis in the

TGFβ/ β2SP/Smad3/CTCF signaling pathway
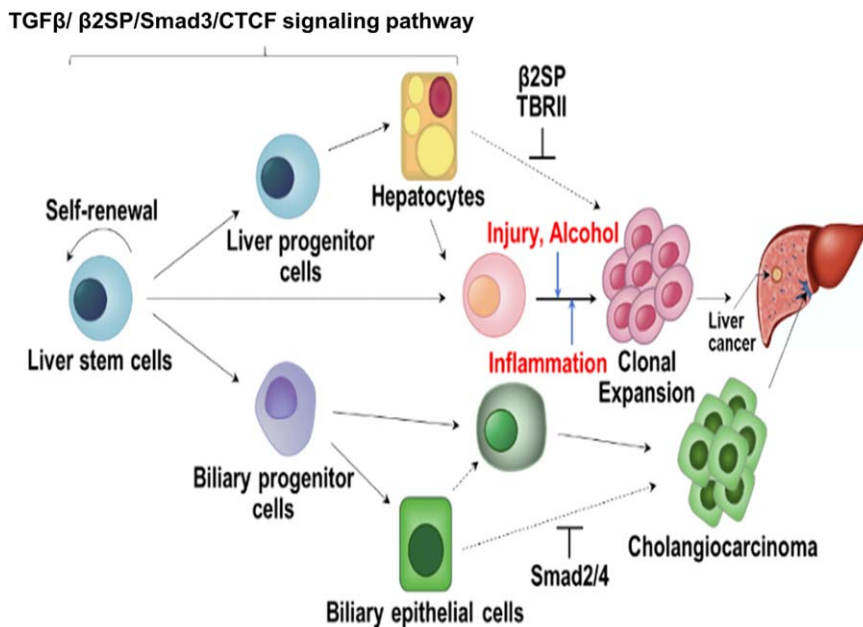
FIG. 3. TGF-β/SMAD/CTCF signaling pathway. The flow chart demonstrates roles of the TGF-β/β2SP/Smad3/CTCF signaling pathway in liver stem cell homeostasis and response to alcohol-induced injury and inflammation.

massive hepatic necrosis group may indicate aberrant TGF-β signaling, which is not observed in the submassive necrosis group where a few hepatocytes are observed.[33] In human liver regeneration, liver progenitor/stem cells are observed along the pericentral vein early in regeneration (before 6 weeks following post transplantation), and later at the portal tracts ($\geq$6 weeks post transplantation)[28] (Fig. 4). Anti-Oct4 and Nanog are observed to label cells as early as the first 1-3 weeks, representing early liver regeneration.[28] These putative progenitor cells carry stem cell markers as well as TGF-β markers, including TBRII and β2SP (TGF-β component Smad3/4 adaptor). Although Smad3 is expressed ubiquitously, we found that the common mediator Smad4 is also expressed in this biliary region, perhaps signifying this population as "committed progenitor cells," and suggesting that TGF-β members play multiple and complex roles in liver stem cell function and in conferring the cell type. Future lineage tracing experiments will identify the cell populations responsible for liver regeneration following injury/damage in the context of TGF-β signaling.

TGF-β, as a pleiotropic cytokine, has been proven to be differentially involved in the regulation of multilineage differentiation of stem cells (Fig. 5), through cross-talk involving the Smad pathway, non-Smad pathways including MAP kinase pathways, PI3K/AKT pathways, and Rho-like GTPase signaling pathways. For instance, TGF-β promotes the differentiation of stem cells into smooth muscle cells,[34-37] chondrocytes,[38-40] neurocytes, hepatic stellate cells, Th17 cells, dendritic cells, and cardiomyocytes.[41] However, TGF-β inhibits the differentiation of stem cells into myotubes,[42] adipocytes, endothelial cells, and natural killer cells.[41] Additionally, TGF-β plays a critical role in bone remodeling and can provide competence for early stages of osteoblastic differentiation, but at late stages, TGF-β acts as an inhibitor.[43,44] In embryonic stem cells (ESCs), another TGF-β family member, bone morphogenetic protein (BMP4) is required for ESC self-renewal through a balanced inhibition of ESC lineage commitment. In mesenchymal stem cells, the BMP signal induces osteoblastic differentiation through Bmpr1b but inhibits osteoblastic differentiation through Bmpr1a.[45] BMP signaling inhibits stem cell activation and expansion in intestinal stem cells.[46] In hematopoietic stem cells, BMP signaling through Bmpr1a restricts stem cell number by controlling the niche size.[47] *In vitro* and *in vivo* studies have demonstrated that Activin/Nodal signaling maintains pluripotency in human pluripotent stem cells[48] and also in mouse epiblast stem cells.[49] Absence of Nodal signaling results in the loss of pluripotency markers and the gain of ectopic neuroectoderm marker expression in the epiblast immediately after implantation.[50,51] BMP4 through Smad1/5/8 and Activin/Nodal through Smad2/3 compete to modulate the expression
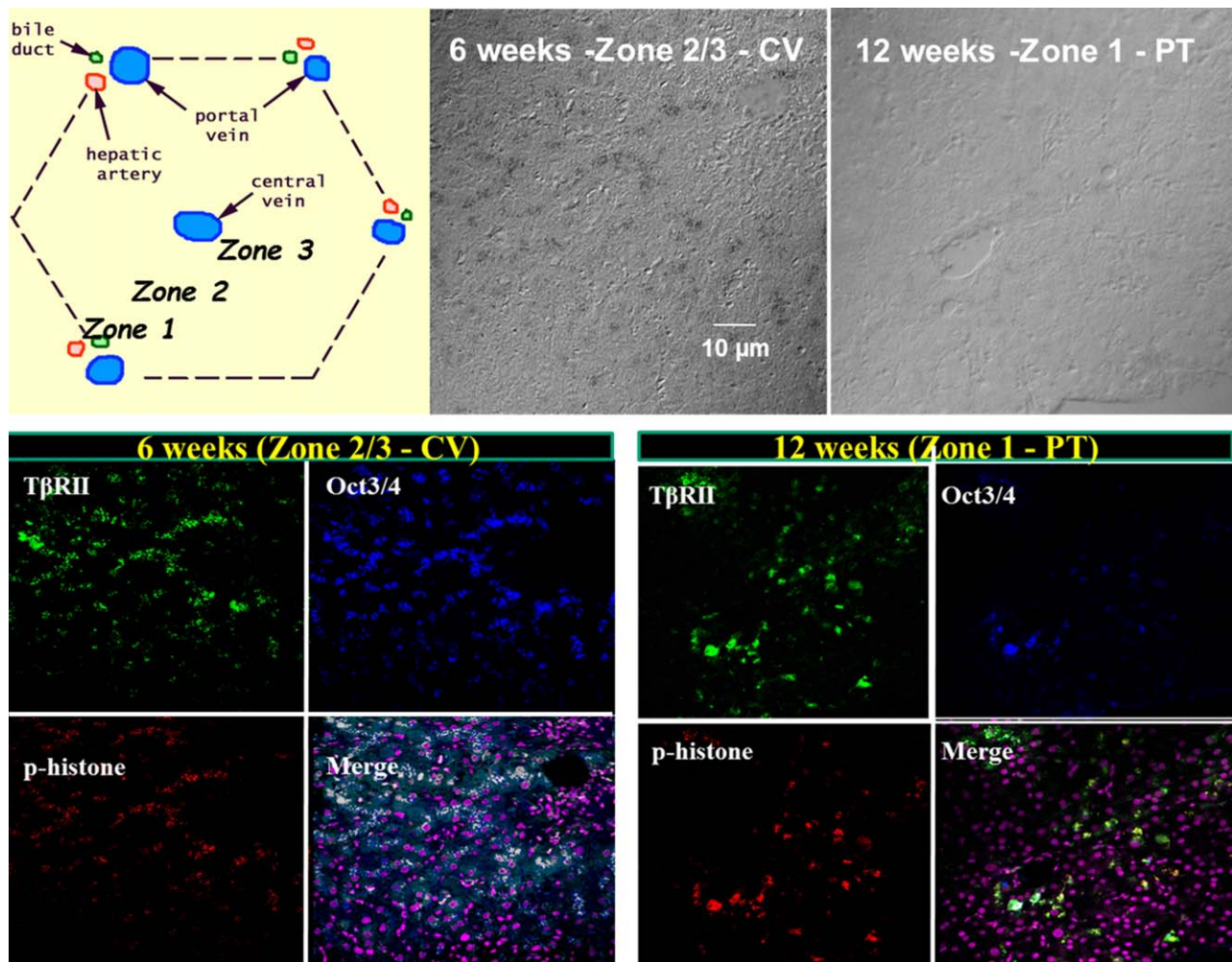
FIG. 4. In human liver regeneration, liver progenitor/stem cells are observed along the pericentral vein early in regeneration (before 6 weeks), and later at the portal tracts (after 6 weeks). As demonstrated colocalization of Oct3/4 and p-Histone, a marker of cell proliferation. Given that hepatocytes primarily drive liver regeneration after acute injury, it is likely that the Oct3/4 and AFP-positive cells are proliferating hepatocytes and expression of these stem cell markers reflects their stem cell–like nature. More importantly, however, Oct3/4 and p-Histone–positive cells also colocalize with β2SP and TBRII at all times, as evidenced in the merged images, where white represents colocalization.

of key pluripotency markers such as Nanog.[52] Activin B and several other genes that are known to be involved in enhancing Activin signaling, such as Wwp2, S100A4, Sulf2, and Inhbb, are also known to be involved in self-renewal of hair follicle stem cells.[53] Recently, TRIM33 has been discovered to act as a signal transducer and direct mediator of transcription in the TGF-β pathway in ESCs. Ligand activation of nodal/Activin receptors induces the formation of TRIM33–Smad2/3 and Smad4–Smad2/3 protein complexes.[54]

Several TGF-β signaling components are tumor suppressors. Inactivation of at least one of these

components occurs in almost all gastrointestinal tumors.[17,55] For instance, TBRII is mutated in up to 30% of colon cancers,[56] TBRI is mutated in 15% of biliary cancers,[57-59] and SMAD4 is deleted in 40%-60% of pancreatic cancers and mutated in gastrointestinal cancer.[60] Loss of β2SP is observed in human HCC.[14,61-63] Evidence from Smad4-knockout mice, which develop head and neck cancers, demonstrates a significant role for Smad4 in promoting genomic stability.[64] Another piece of evidence from studies of liver regeneration that implicates TGF-β pathway in cancer involves vitamin D deficiency. Vitamin D supplementation is essential for TGF-β pathway member expression

	Wnt/Axin	CD133	EpCAm	Sox9	LGR5	TGF-β, Smads/β2SP
• Mouse knockouts	X	X	X	X	X	✓
• Stem cell: self renewal, differentiated progeny	✓	M✓	M✓	✓X	✓	✓
• CSCs Tumors: self renew generate diverse cell type	M✓	M✓	MX	X	✓	✓
• Animal models, liver regeneration	✓	✓	✓	X	✓	✓
• Lineage studies	✓	✓	✓	X	✓	X
• Repopulation	✓	X	X	X	✓	X
• Human Liver Regeneration/Genetic Studies	X	X	X	X	X	✓
	✓	X	X	X	X	✓
• Human Genomics						

FIG. 5. Summary of mouse knockout and lineage studies on candidate molecules in liver stem cells/cancer stem cells. Data were obtained from the Mouse Genome Informatics Database. Wnt/Axin: no liver phenotypes were observed, but 65 other phenotypes observed, including limb, kidney, and intestine. β-catenin: 341 phenotypes were also observed, including bone and intestinal abnormalities. No liver phenotypes were observed. CD133: phenotypes observed included retinitis pigmentosa and retinal degeneration (137). EpCam: no liver abnormalities were observed, but small intestinal and trophoblast abnormalities were observed. Sox9: No liver phenotypes were observed, but 184 other phenotypes were observed, including bone, pancreas, and eye abnormalities. LGR5: 12 phenotypes were observed, including intestinal abnormalities, distended abdomen and neonatal death. In contrast, TGF-β members display definitive foregut-liver lineage and multiple liver cancer phenotypes. Smad4: 154 phenotypes were observed including ectoderm, mesoderm, no primitive foregut and multiple gastrointestinal cancers. Smad2/Smad3, β2SP: multiple liver and gastrointestinal abnormalities, and cancers were observed. (M = marker).

levels. β-catenin activation in fibrotic/cirrhotic human liver tissues and vitamin D deficiency promotes tumor growth in the context of Smad3 disruption, potentially through the regulation of TLR7 expression and β-catenin activation. Whole genome and transcriptomic analyses of somatic mutations and alterations in genes involved in vitamin D metabolism, vitamin D–related genes, and the TGF-β superfamily using The Cancer Genome Atlas database of 147 patients with liver cancer revealed positive correlation between inactivating somatic mutations for vitamin D–related genes and the TGF-β pathway and plays a critical role in liver tumorigenesis.[65] In addition, TGF-β1 inhibits telomerase activity. Conversely, TGF-β1–induced arrest of cell growth can be overcome by the activation of human TERT, the protein catalytic subunit of telomerase.[66] Telomerase activation and maintenance is important for malignant transformation from normal cells.[67] Keratinocytes cultured from TGF-β1–null mice have marked genomic instability that could accelerate tumor progression.[68] More recently, studies have been conducted in Smad4 conditional knockout mice that develop head and neck cancers, where Smad4 has been postulated as a "guardian of the genome" through regulation of the Fanconi anemia/Brca (Fanc/Brca) DNA repair pathway.[64,69] Interestingly, the development of HCCs in β2SP heterozygote mutants establishes β2SP as a nontraditional and functional tumor suppressor. Spectrins have been observed to associate with Fanconi proteins (G and D) as well as with DNA interstrand cross-links.[70,71] Similarly, by virtue of its involvement in Smad3/4 localization and subsequent activation of Smad3/4, β2SP may enhance TGF-β tumor suppressor function. Also, TGF-β–deficient β2SP mutant mice are highly susceptible to alcohol injury, marked by an abnormal response to DNA cross-linking repair. Taken together, these studies indicate TGF-β as a potential processor of genomic stability through modulation of the Fanc pathway at interstrand crosslinks, yet clear mechanisms remain to be elucidated.

TGF-β in Liver Cancer Stem Cells

Pathways involved in stem cell transformation into gastrointestinal cancers remain largely undefined and a

FIG. 6. BWS is a human cancer stem cell overgrowth disorder with an estimated prevalence of 1 in 14,000. The syndrome includes heterogeneous features such as organomegaly and adrenal cytomegaly (a hallmark characteristic). Sptbn1$^{+/-}$/Smad3$^{+/-}$ mice generated in the laboratory were phenocopies of BWS patients. Sptbn1$^{+/-}$/Smad3$^{+/-}$ mice demonstrated microfacies and the classic metopic ridge frequently associated with human BWS and a high risk of cancers that include HCC.

black box. A recent discovery reports that TGF-β–deficient mutant mice closely resemble a cancer stem cell disorder, including characteristic ear abnormalities and adrenal cytomegaly. Beckwith-Wiedemann syndrome (BWS) is a human stem cell overgrowth disorder with an estimated prevalence of 1 in 14,000[72] (Fig. 6). The syndrome includes heterogeneous features such as organomegaly and adrenal cytomegaly (a hallmark characteristic)[73] (Fig. 7). BWS is associated with an 800-fold increased risk of childhood neoplasms, and can develop multiple tumor types within the same organ simultaneously, an example including the co-occurrence of a mesenchymal hamartoma, capillary hemangioma hepatoblastoma, and cholangiocarcinoma within the liver of one patient.[74,75] These events are suggestive of the multipotentiality of neoplastic transformation and imply dysfunctional processes as stem cells differentiate into mature adult cell types.[76] Mechanistic insight into downstream effector pathways which lead to stem cell transformation and an integrated analysis from mouse models to human

disease for BWS and associated cancers remain only partially defined.

These new studies demonstrate that TGF-β induces chromatin insulator CCCTC-binding factor (CTCF) which facilitates TGF-β–mediated repression of TERT transcription via interactions with β2SP and SMAD3. This regulation is abrogated in TGF-β–defective mice and BWS, resulting in TERT overexpression. Tert induction in Sptbn1$^{+/-}$/Smad3$^{+/-}$ mouse embryonic fibroblasts suggests that dysregulated telomerase expression may be part of the molecular basis of tumor development in BWS patients. These results show that recruitment of the SMAD3/β2SP/CTCF complex at the TERT promoter resulting in tumorigenesis is dependent on the TGF-β signaling pathway.[14] Moreover, activation of the SMAD3/β2SP/CTCF complex on the TERT promoter region may cooperate with MYC activation. MYC activation and telomerase dysfunction have been shown to play prominent roles in early HCC initiation.[77] Therefore, the TGF-β–mediated β2SP/SMAD3/CTCF complex
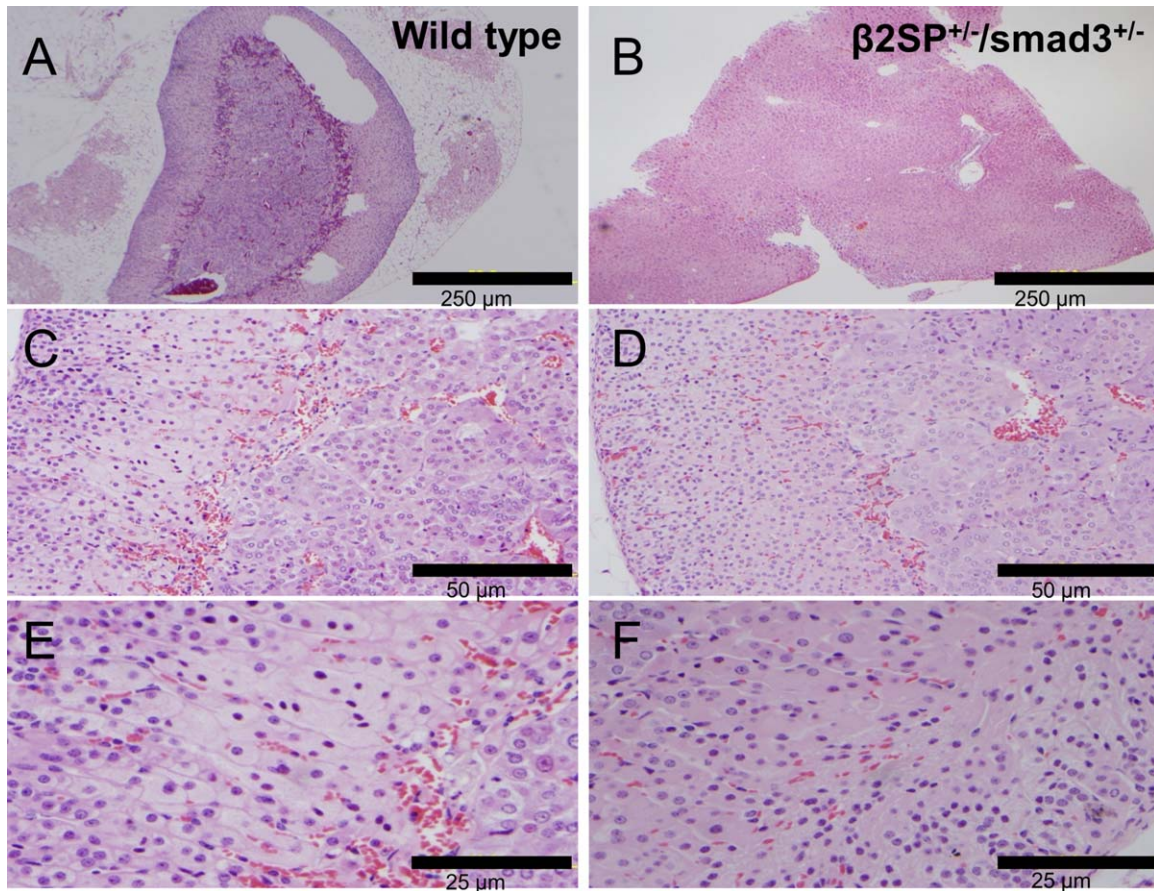
FIG. 7. Identification of cytomegaly of the fetal cortex of the adrenal glands in a $\beta2SP^{+/-}/Smad3^{+/-}$ mouse (B, D, F) compared with a wild-type mouse (A, C, E).

regulates telomerase activity and is part of a pathway that suppresses the switch to tumorigenesis in BWS-associated cancers. Identifying similar key mechanisms through such mouse models that reflect the human disease could lead to effective new treatment strategies.

TGF-β in an Invasive Cancer Stem Cell Model

How chronic inflammation modulates stem cells and cancer remains only partially defined, and is another black box. Chronic inflammation, often associated with liver injury, leads to secretion of cytokines, chemokines, free radicals, and other DNA-damaging molecules, thereby changing the hepatic microenvironment.[78] Persistent inflammation during this long-term process leads to an expansion of hepatic stem and progenitor cells that accumulate genetic and epigenetic alterations.

Thus, the highly inflamed liver immune microenvironment is a major driver of the transformation of normal liver stem cells (LSCs) to highly metastatic cancer stem cells (CSCs).

Molecular mechanisms that link chronic inflammatory responses with tumor initiation have been studied extensively.[79,80] Among numerous proinflammatory factors, interleukin-6 (IL-6) is the most prominently elevated in almost 40% of liver cancer patients, suggesting that IL-6 is associated with HCC progression.[81-87] The TGF-β pathway induced IL-6 secretion may confer chemotherapeutic resistance in HCC.[32] IL-6 is required for the priming of hepatocytes to leave their quiescent state (G0) and enter a prereplicative phase (G1), and transcriptionally up-regulates an array of genes during liver growth.[88] A recent study showed that signal transducer and activator of transcription 3 (STAT3), following its IL-6–mediated activation, binds to the promoter element of CD133 to induce
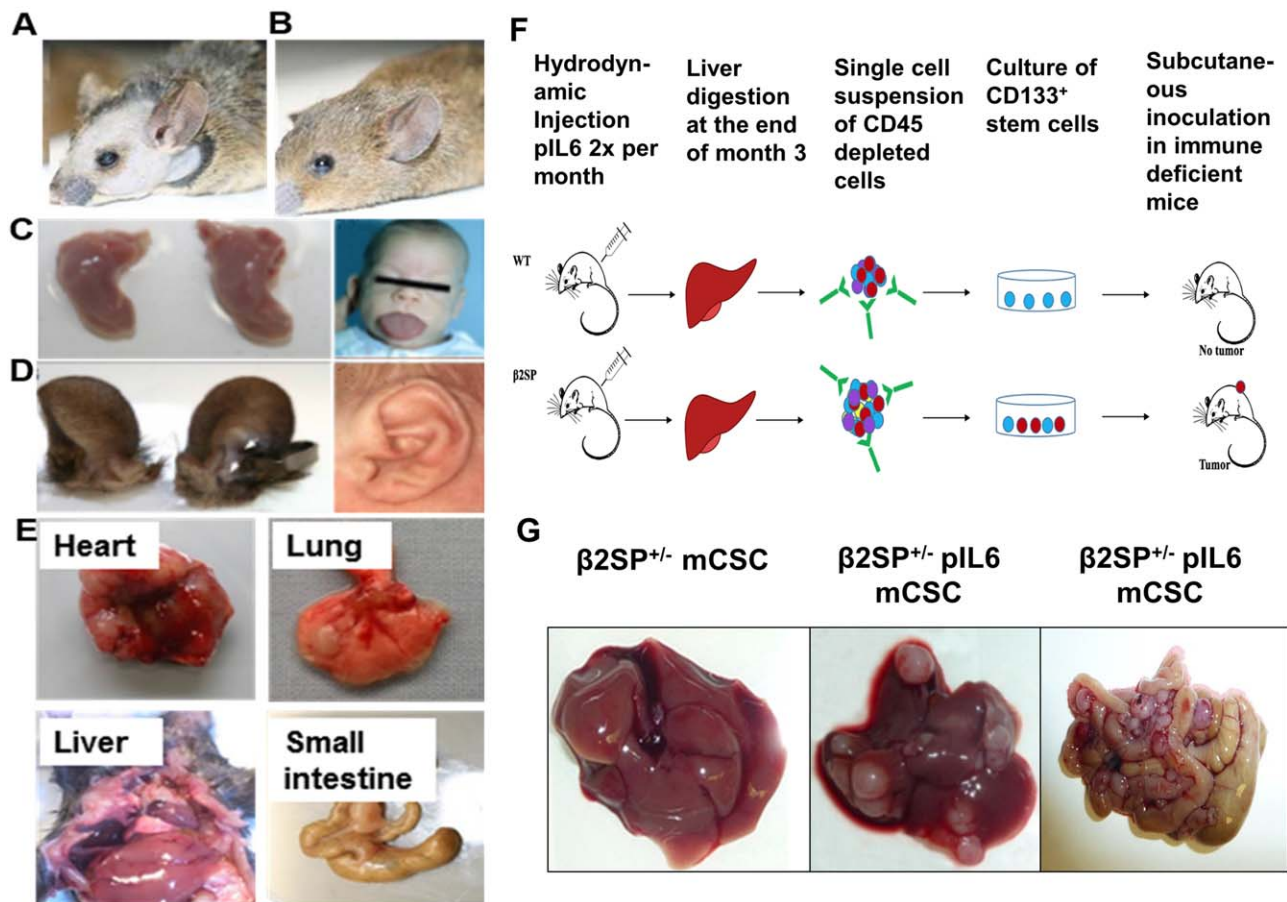
FIG. 8. Cancer stem cell models of HCC–TGF-β, IL-6. (A) β2SP$^{+/-}$/Smad3$^{+/-}$ mouse phenocopy of BWS compared with (B) wild-type mouse. (C) Macroglossia and (D) abnormal anterior ear creases in β2SP$^{+/-}$/Smad3$^{+/-}$ mice and BWS patients. (E) β2SP$^{+/-}$/Smad3$^{+/-}$ mice spontaneously develop liver and gastrointestinal cancers by 12 months of age. (F) Schematic representation of chronic treatment with IL-6–induced cancer stem cells in β2SP$^{+/-}$ mice. (G) Orthotopic invasive tumor development after stem cell inoculation shown in panel F.

HCC progression.[89] Liver cancer patients with high levels of CD133 expression have shorter overall survival and higher relapse rates than those with low levels of CD133 expression.[90] IL-6–mediated inflammation programs, constitutive activation of the TGF-β–activated kinase 1 (TAK1)/nuclear factor kappa B (NF-κB) signaling cascade in CD133$^+$ LSCs, and this programming, interacts with deficient TGF-β signaling, thereby accelerating the transformation of normal LSCs to metastatic CSCs.

CD133$^+$ LSCs derived from preneoplastic livers of TGF-β–deficient β2SP$^{+/-}$ mice treated with IL-6 were highly tumorigenic and metastatic and exhibited nuclear localization of Twist and Slug (markers of epithelial-mesenchymal transition) and constitutive activation of NF-κB[91] (Fig. 8). NF-κB was activated by TAK1 (MAP3K7), which is associated with poor survival in HCC and IL-6 expression. Hepatocyte-specific deletion of TAK1 in mice activates the TGF-β signaling pathway to induce spontaneous inflammation, fibrosis, and, eventually, hepatic tumorigenesis.[92] Overall, this reciprocal regulation between β2SP and IL-6 in the programming of liver CSCs provides insight into the mechanism by which normal stem cells transform into EMT-positive CSCs.[93] Therefore, these studies demonstrate that there exists a reciprocal cross-talk between TGF-β signaling and IL-6–driven inflammation in preneoplastic liver tissues and defining the mechanisms by which the loss of TGF-β/β2SP regulates the transition of hepatic stem cells to cells with EMT phenotypes in an inflammation-driven hepatic immune environment.

Discovery of this mechanistic insight will improve modern therapeutic approaches to eliminate metastatic tumor stem cells at an early stage before tumor initiation and will shed new light on the black box of mechanisms of stem cell transformation. Furthermore, this discovery will provide a scientific basis for a specific targeted therapy of metastatic CSCs for preventing invasion, resistance, and relapse.

The Phases of Liver Regeneration

In the adult liver, mature hepatocytes seldom proliferate and have a life span of over a year.[94] After partial hepatectomy, however, proliferation of the normally quiescent hepatocytes and cholangiocytes, followed by proliferation of the hepatic stellate cells and endothelial cells quickly restores the liver to its original mass. In the rodent model, DNA synthesis starts 12 to 16 hours after the standard partial hepatectomy (PHx) and peaks at 24-48 hours. The original organ mass is almost restored 3-7 days postresection, and by 3-4 months in humans.[95,96] Liver regeneration therefore represents an example of precisely controlled initiation and synchronized cell proliferation *in vivo*, in which normally quiescent hepatocytes exit G0, reenter the cell cycle, and undergo one or two rounds of replication, with restoration of liver mass and function.[94,97,98] The initiation step is characterized by priming of quiescent hepatocytes by factors such as tumor necrosis factor α (TNF-α), IL-6, and nitric oxide. These cytokines are released within minutes of partial hepatectomy from nonparenchymal liver cells and induce hepatocytes to synthesize further acute phase proteins—mainly protease inhibitors—through activation of hepatocyte DNA-binding proteins.[99,100] Therefore, more than 100 immediate early genes are activated by latent transcription factors at the transition between G0 and G1. For instance, within minutes, specific transcription factors such as NF-κB, STAT3, and AP1 are rapidly activated in remnant hepatocytes, as are intracellular signaling pathways such as mitogen-activated protein kinase, phosphorylated extracellular signal-regulated kinases, and Jun amino-terminal kinase.[101-103] The result is an induction of hepatocytes to become sensitive to growth factors and competent for replication.

The proliferation step arises when hepatocytes enter the cell cycle as G1 phase and are stimulated by complete mitogens including hepatocyte growth factor (HGF). HGF increases 10- to 20-fold in the plasma within the first 3 hours after PHx and activates the HGF receptor cMet within 30-60 minutes.[104,105] Similarly, plasma concentration of TNF-α, IL-6, epidermal growth factor, and TGF-β1 increase within 1-2 hours after PHx. These hepatomitogens, together with co-mitogens such as norepinephrine and potentiating factors such as insulin, induce hepatocytes to override the mitogen restriction point at two-thirds of the G1 phase and progress into DNA synthesis. These factors induce cyclins and cyclin-dependent kinases that play critical roles in cell cycle progression.[106-108] Intracellularly, β-catenin and the Notch1 intracellular domain translocate to hepatocyte nuclei within 15-30 minutes, and enhanced activation of STAT3 and NF-κB within 1 hour contributes to activation of signaling pathways leading to cell cycle progression.

Cell cycle progression, regulated by the sequential formation, activation, and inactivation of complexes composed of cyclin-dependent kinases (Cdks) and cyclins proceeds in a synchronized pattern following PHx. In mid- to late G1, phosphorylation of the retinoblastoma protein by Cdk4/6-cyclin D complexes initiates the cell cycle and mediates the G1/S-phase transition.[109] Cdk2 then successively associates with cyclins E and A, completes phosphorylation of retinoblastoma protein, promotes activation of the DNA replication machinery, and regulates centrosome duplication, completing transition into S-phase. Cdk1, in association with cyclins A and B, is then essential for entry and exit from mitosis. Cyclin D1 has been shown to be activated by 6 hours and maximal levels of Cdk4 are present at 24 hours after PHx in rats.[110] Meanwhile, Cdk1 is sharply induced between 18 and 24 hours, followed by a transient decrease, before another increase at 30 hours post-PHx in rats.[111] Regeneration is complete, when an appropriate functional size is reached. TGF-β plays a prominent role in this phase through inhibition of DNA synthesis in regenerating hepatocytes.

Temporal and Spatial Fluctuations of TGF-β Associated Members in Liver Regeneration: An Insight Into Their Function

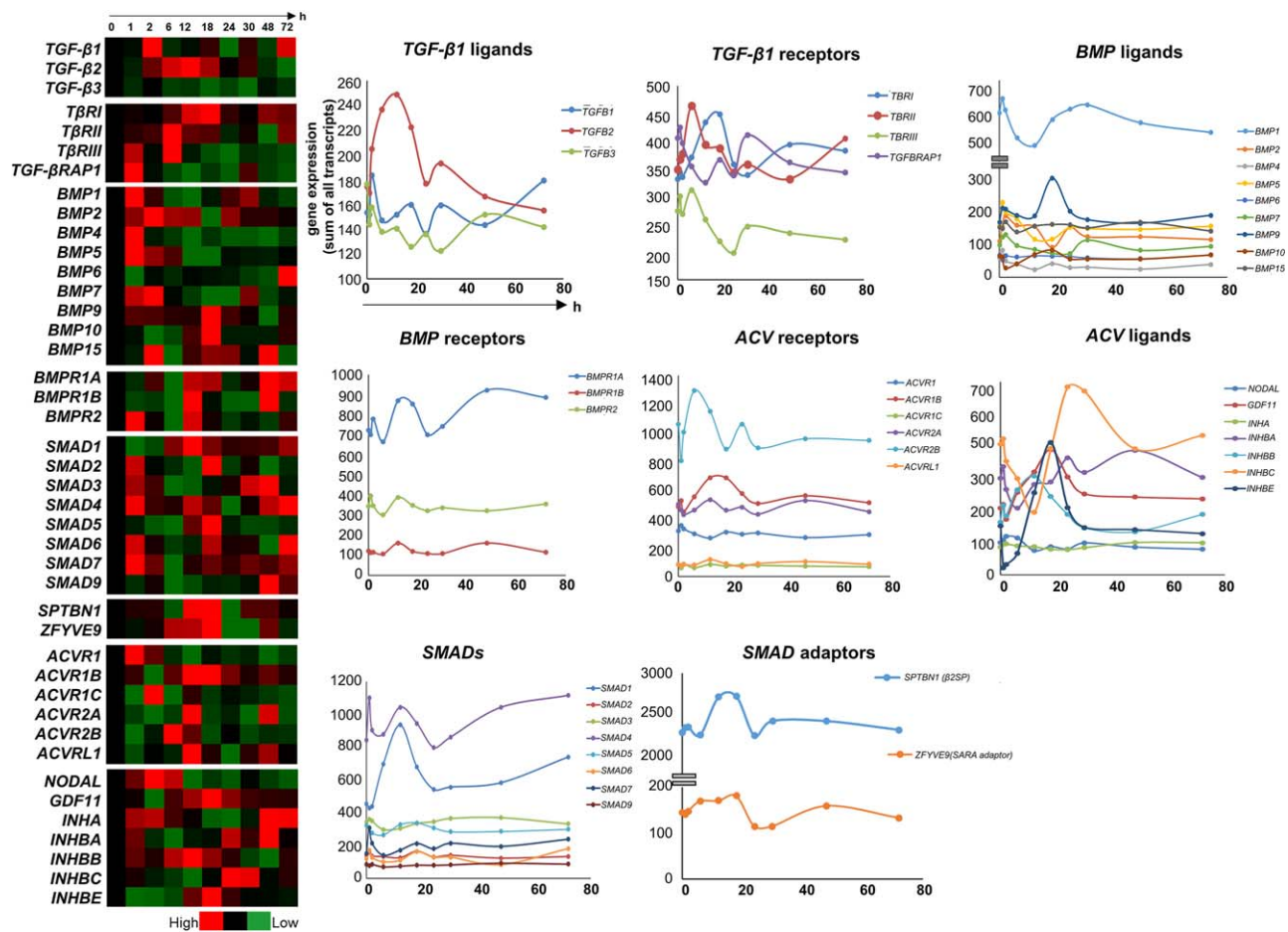TGF-β signaling has been shown to reversibly inhibit the proliferative response following partial

FIG. 9. TGF-β–associated genes had a characteristic pattern of mRNA expression during a 72-hour period after PHx. Core TGF-β pathway–related genes were evident in liver regeneration.

hepatectomy.[112] TGF-β1 levels are raised in the first 2 hours after PHx, and expression levels of downstream Smads, phospho-Smad2, Smad2, and Smad4 are similarly elevated.[95,113] Concomitant up-regulation of TGF-β inhibitory proteins, SnoN and Ski, and a down-regulation of the TGF-β receptors, allows hepatocytes to transition from G1 to S phase.[114] TBRII-conditional knockout mice demonstrate accelerated proliferation and an increased liver mass to body weight ratio after PHx.[115]

TGF-β is primarily synthesized by stellate cells, a cell type that resides within the space of Disse (perisinusoidal space) in recesses between hepatocytes. There are approximately 2-20 stellate cells per 100 hepatocytes, and these are activated during liver injury and become myofibroblasts that produce extracellular matrix, leading to progression of fibrosis and liver

disease in the aberrant state. TGF-β released from these stellate cells has a paracrine effect on hepatocytes. TGF-β perturbations occur primarily in hepatocytes or stellate cells, depending on which cell type is dominantly undergoing proliferative changes after liver injury. Each isoform of TGF-β has its own characteristic pattern of messenger RNA (mRNA) expression (which is assumed to have a high degree of correlation to secreted protein levels[116]) during a 72-hour period after PHx (Figs. 9 and 10).[116] The early phase of TGF-β1 expression (2- to 6-hour time point) precedes the major peak of hepatocyte growth, while the 48- to 72-hour time point coincides with the decrease in liver cell turnover.[116] The β1 isoform has an early peak at 2 hours, but then decreases and spikes again between 48 and 72 hours in hepatocytes. This suggests that the β1 isoform probably plays two different roles in liver

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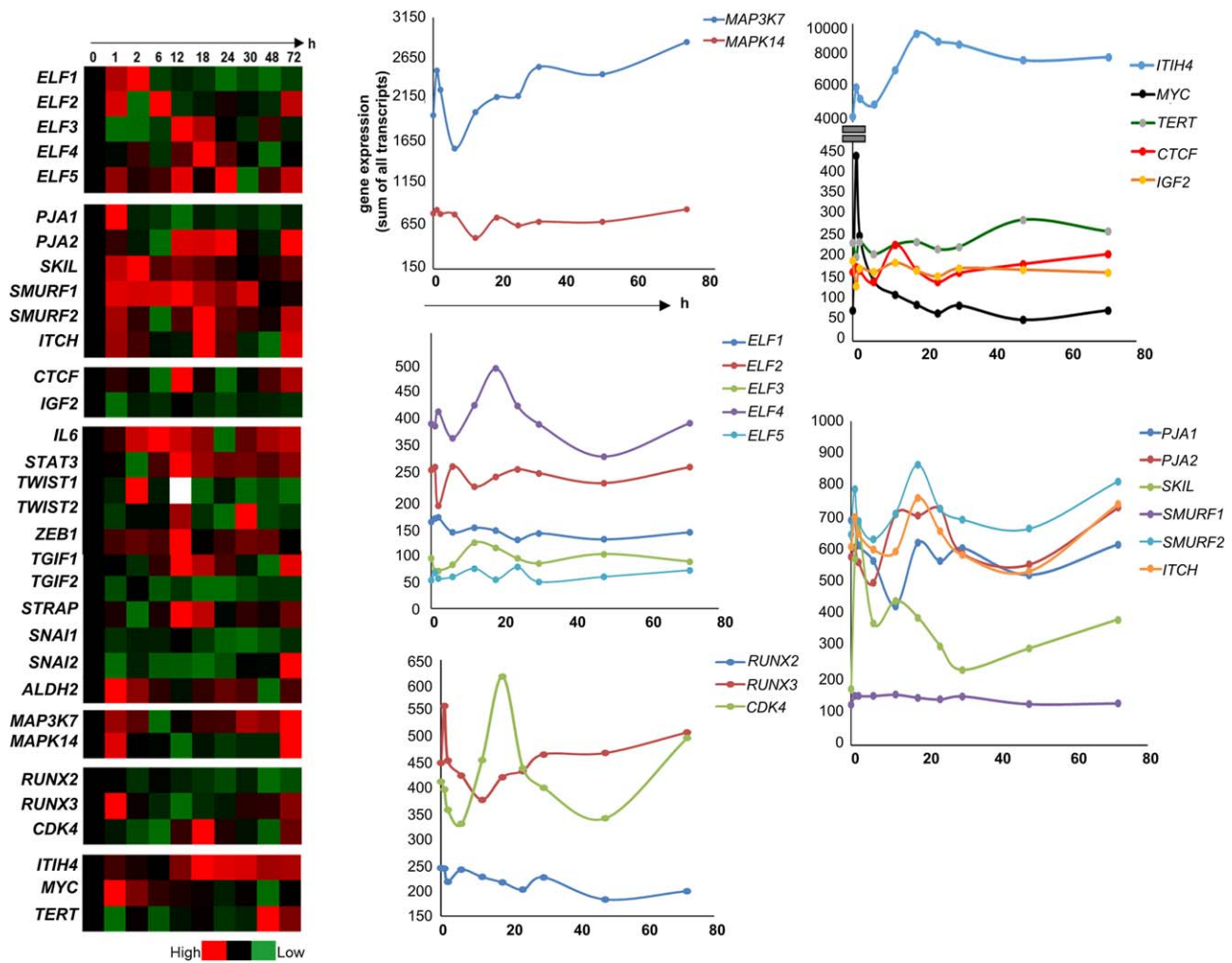
FIG. 10. mRNA expression of other TGF-β pathway–related genes was evident in liver regeneration during a 72-hour period after PHx.

regeneration: a major role both during the initial phase as liver regeneration progresses to DNA synthesis, as well as role in the later, termination phase of liver regeneration after injury. The $\beta2$ isoform is increased in all liver cell types at hour 6, but at the later time point, the hepatocyte fraction shows a further increase, whereas the nonparenchymal cell fractions decline. The $\beta3$ isoform shows major increases in all cell fractions at the early time point, but only the hepatocyte fraction maintains this increase at the later time point.[116,117]

Levels of TBRI and TBRII involved in TGF-β signaling are decreased in the early liver regeneration phase while TBRIII and TGF-βRAP1/TRAP1 which are known to be inhibitors of TGF-β signaling[118,119]

are elevated. These expression levels are reversed in correlation to TGF-β peaks in the 6- and 72-hour phases. Bone morphogenetic proteins (BMPs) are members of the TGF-β family and act as constitutively expressed repressors of regeneration. Consistent with this hypothesis, BMPs are expressed in the initial phase within 2 hours and then down-regulated as TGF-β spikes.[116,120] In the normal liver, strong BMP2 expression is observed around the central and portal veins. The observed down-regulation of BMP2 in rat liver following partial hepatectomy suggests that such down-regulation may be necessary for hepatocyte proliferation.[121] Mice driven to maintain BMP4 expression in the liver, display inhibited hepatocyte proliferation and restoration of liver mass after

hepatectomy, suggesting that reduced BMP4 is necessary for normal regeneration.[120] Consistent with this finding, the BMP receptors also follow an inverse pattern of up-regulation compared with TGF-β activation and are mostly up-regulated in the anti-proliferative/termination phase.[116]

Hepatocyte-specific deletion of the BMP receptor Activin receptor-like kinase 3 enhances regeneration.[120] The BMP4 antagonist Noggin has also been reported to enhance regeneration. BMP7 expression is absent in liver; however, neutralization of circulating endogenous BMP7 results in significantly impaired regeneration of the liver after partial hepatectomy, whereas therapeutic administration of recombinant human BMP7 significantly enhances liver regeneration. BMP9 stimulation of cultured hepatocytes inhibited proliferation. Constitutive expression of low levels of BMP9 stabilizes hepatocyte function in the healthy liver. Acute liver injury caused by partial hepatectomy results in transient down-regulation of hepatic BMP9 mRNA expression and following HSC activation, endogenous BMP9 levels again increase.[122] Regulation of Activin signaling through receptors is another major factor determining liver regeneration after liver injury. Activins inhibit DNA synthesis in hepatocytes and Activin and their receptors are initially down-regulated and later induced between 24 and 72 hours after the proliferation slows down.[123] Likewise, Inhibins which have biological effects directly opposite to those of Activins are induced in the initial phase, down-regulated when Activins are up-regulated, and elevated again in the later phase when activin expression is reduced.[116]

Smad proteins are intracellular effectors of TGF-β signaling and transduce signals from TGF-β superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. Partial hepatectomy stimulates a strong regenerative response with elevated expression of Smad2/3 phosphorylation in the first 2 hours followed by IL-6, TNF-α, and STAT3 induction 24 hours post-PHx in both hepatocytes and nonparenchymal cells.[116,124] Smad3 deficiency leads to reduced hepatocyte proliferation 42 hours post-PHx, a process that correlated with and was preceded by significant reductions in IL-6 expression and STAT3 phosphorylation. STAT3, upon activation by a number of factors including IL-6, regulates cell survival and proliferation and liver regeneration and loss of STAT3 in hepatocytes reduces their proliferation early during regeneration after PHx.[125,126]

Among the other SMADs, suppression of SMAD1, SMAD5, and SMAD9 is known to repress liver regeneration.[127] SMAD6 inhibits Wnt/β-catenin signaling and suppresses the growth and self-renewal of hepatic progenitor cells.[128] Adaptors such as β2 spectrin (β2SP) are involved in hepatocyte proliferation through the interaction of TGF-β/Smad and PI3K/AKT signaling.[129] β2SP deficiency results in dysfunctional hepatocyte cell cycle progression and delayed liver regeneration at 48 hours after PHx. This defect is mediated by dysfunctional expression of cell cycle proteins and by increased DNA damage.[27] Spatial and temporal expansion of TBRI and β2SP expression occurs as regeneration proceeds. TBRI and β2SP expression increases gradually in the initial phase, until approximately 18 hours after hepatic injury, and then decreases.[116] The spatial expansion of TBRI and β2SP proceeds from periportal to pericentral areas of lobules, suggesting an important role for the TGF-β signaling molecules in liver regeneration in response to liver injury.[28] An interesting transition between PRAJA (PJA1), an E3-dependent ubiquitin ligase of β2SP, and β2SP proteins, occurs at 6 hours postinjury, with expression of PJA1, and β2SP predictably inversely proportional to each other. At 6 hours, PJA1 levels begin to decrease, allowing the up-regulation of β2SP on a background of TGF-β expression, which is already high compared with expression in normal liver. As PJA1 expression continues to decrease over 6-12 hours postinjury, β2SP remains accumulated in the cells.[130] Potentially targeting similar E3 ligases that are activated in the setting of loss of TGF-β tumor suppressor activity, could provide attractive new therapeutics for HCC.

Serial transplantation experiments have shown that hepatocytes have a near infinite capacity to proliferate.[94,131,132] When mature hepatocytes and cholangiocytes are damaged or inhibited in their replication, however, a reserve compartment of hepatic progenitor cells are activated.[133] In human liver donor transplant recipients, early on within the first 6 weeks, expansion of cells expressing stem cell markers Oct3/4, AFP, and TGF-β members is observed in zone 1, zone 2,[28] and surprisingly also zone 3 (central vein)[134-136] and appears to give rise to hepatocytes. These studies suggest that in submassive hepatic necrosis with intact zones 1 and 2, cells express stem cell markers and potentially lead the regenerative process.[28,33] The activation of the stem cell compartment, originally referred to as a "ductular reaction" in humans and "oval cell reaction" in rodents, is observed in circumstances of prolonged necrosis, cirrhosis, and chronic inflammatory liver diseases. In summary, rodent studies have led

to the identification of the three stem cell compartments displayed by markers of the Wnt/Axin/Lgr5 family, EpCam, and CD133. Yet mouse mutants of the single gene knockouts do not reveal significant liver pathology (Fig. 5). Mouse knockouts, human liver regeneration, and functional studies thus reveal a pivotal role for TGF-β in suppressing cancer stem cells, as well as modulating processes of liver regeneration and fibrosis.[27,28] However, exactly how it is defined temporally with existing stem cell markers such as Axin1, Lgr5, Fancd2, and CD133 remains to be explored. Importantly, the studies provide a key role for the TGF-β pathway in suppressing cancer stem cells, and the loss of TGF-β signaling in these cells could lead to better identification of these cells, as well as prediction of tumor behavior and ultimately lead to precise targeting of cancer stem cells in this lethal cancer.

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