Loss of STAT5 causes liver fibrosis and cancer development through increased TGF-β and STAT3 activation

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The molecular mechanisms underlying the development of hepatocellular carcinoma are not fully understood. Liver-specific signal transducer and activator of transcription (STAT) 5A/B–null mice (STAT5-LKO) were treated with carbon tetrachloride (CCl4), and histological analyses revealed liver fibrosis and tumors. Transforming growth factor (TGF)–β levels and STAT3 activity were elevated in liver tissue from STAT5-LKO mice upon CCl4 treatment. To define the molecular link between STAT5 silencing and TGF–β up-regulation, as well as STAT3 activation, we examined STAT5-null mouse embryonic fibroblasts and primary hepatocytes. These cells displayed elevated TGF–β protein levels, whereas messenger RNA levels remained almost unchanged. Protease inhibitor studies revealed that STAT5 deficiency enhanced the stability of mature TGF–β. Immunoprecipitation and immunohistochemistry analyses demonstrated that STAT5, through its N-terminal sequences, could bind to TGF–β and that retroviral-mediated overexpression of STAT5 decreased TGF–β levels. To confirm the in vivo significance of the N-terminal domain of STAT5, we treated mice that expressed STAT5 lacking the N terminus (STAT5–ΔN) with CCl4. STAT5–ΔN mice developed CCl4-induced liver fibrosis but no tumors. In conclusion, loss of STAT5 results in elevated TGF–β levels and enhanced growth hormone–induced STAT3 activity. We propose that a deregulated STAT5–TGF–β–STAT3 network contributes to the development of chronic liver disease.

Hepatocellular carcinomas (HCC) are a major cause of cancer death and mostly develop as a result of advanced liver fibrosis. Studies have linked the development of HCC with a methylation-induced silencing of SOCS (suppressor of cytokine signaling) genes, which in turn leads to elevated activation of signal transducer and activator of transcription (STAT) 3 (1) or mutations of Axin1, a negative regulator of Wnt signaling (2). However, the mechanisms by which liver fibrosis induces cancer remain elusive.

It is well known that TGF-β is an important cytokine in these fibrotic processes (3, 4) and that excess TGF-β production is a key problem for the treatment of liver diseases as it triggers liver fibrosis (4). Three different gene products, TGF-β1, -β2, and -β3, have been cloned from mammalian tissues (5, 6) and mature TGF-β proteins are composed of two 12.5-kD polypeptides. They are derived from 55-kD polypeptides, which dimerize upon synthesis followed by processing and secretion (7). Most studies describing biological effects of TGF-β have examined how it affects targeted cells. In this paper, we have focused on intracellular processing of the TGF-β complex before secretion. Transformation of hepatocytes, but not of hepatic stellate cells (HSC) or Kupffer cells (KC), leads to cancer as a result of liver fibrosis. Although hepatocytes have not been considered a source of TGF-β, we can hypothesize that intracellular synthesis and modification of TGF-β in these cells is involved in cancer development.
The transcription factors STAT5 and STAT3 promote cell cycle progression and suppress apoptosis, thus contributing to cellular transformation. Notably, activated STAT3 can mediate cellular transformation (8) and has been detected in primary tumors including HCC (1, 9–11). Although STAT5 has been linked to tumorigenesis (12–15), it can also act as a tumor suppressor by inhibiting expression of NPM1-ALK (16). As for solid tumors, the role for STAT5 is controversial (17–19). To explore the role of cytokine signaling through STAT5 in liver fibrosis and cancer, we deleted the Stat5 locus specifically in hepatocytes using Cre-mediated recombination. Using this experimental approach, we discovered a relationship between STAT5, TGF-β, and STAT3 in the pathogenesis of chronic liver disease.

RESULTS
Progression of liver fibrosis in hepatocyte-specific STAT5-null mice

Treatment of mice with carbon tetrachloride (CCL4) induces liver fibrosis and is an accepted model to mimic human disease. To examine the significance of cytokine-STAT5 signaling in the development of liver fibrosis, control mice and mice from which the Stat5 locus had been deleted in hepatocytes using Cre-mediated recombination (STAT5-LKO) were treated for 8 wk with CCL4. Loss of STAT5 did not affect the sensitivity of primary hepatocytes to CCL4 and the slight increase of cell death was not significant (Fig. S1, A and B). Before treatment, there were no histological differences between mice from both groups based on silver and Azan staining (Fig. 1, A and B, top). The reticular fibers (Fig. 1, A and B, arrows) extended and developed into the interlobular septum in STAT5-LKO mice, whereas few reticular fibers deposited in the periportal tissue space in controls (Fig. 1 A, bottom). To evaluate fibrotic changes, we also performed Azan staining to detect collagen accumulation and identified the formation of nodules (Fig. 1 B, arrows) in STAT5 mutant mice (Fig. 1 B, bottom). In agreement with these observations, the content of hepatic collagen was higher in STAT5-LKO mice after treatment (Fig. 1 C). These histological analyses and hepatic collagen assays revealed that STAT5 mutant mice displayed more advanced liver fibrosis than control mice.

TGF-β levels and STAT3 activity are elevated in STAT5-LKO mice after CCL4 treatment

The deletion of the Stat5a/b locus in hepatocytes was confirmed by Western blot analyses and STAT5A/B levels were reduced by ~80% (Fig. 1 D). TGF-β (mature TGF-β) levels were more profoundly induced in STAT5-LKO mice after CCL4 treatment compared with controls. Latent TGF-β binding protein (LTBP) levels were reduced in STAT5-LKO mice after CCL4 treatment and to a lesser extent in controls. This reduction of LTBP might be the result of an increased secretion of mature TGF-β (20, 21). The expression levels of other fibrogenesis-related factors, such as platelet-derived growth factor α/β and TGF-β pseudo receptor Bambi, were examined and no differences were observed between control and STAT5-LKO mice (Fig. S2).

Liver fibrosis is reported to associate with acquired growth hormone (GH) resistance because of low GH receptor levels (22, 23). Although strong activation of STAT5 was observed in hepatocytes of control mice upon stimulation with GH, reduced activation was detected after CCL4 treatment (Fig. 1 E), suggesting that these cells lose their hormonal response during early stages of fibrosis. STAT5 levels and activation were greatly reduced in STAT5-LKO mice before and after GH stimulation irrespective of CCL4 treatment. GH receptor levels were largely independent of GH and CCL4 treatment.

We have recently reported that in the absence of STAT5, other STAT members, especially STAT3 and STAT1, could be recruited to receptors normally engaged by STAT5, which in turn would result in their aberrant activation (24, 25). As shown in Fig. 1 E, GH weakly induced tyrosine phosphorylation of STAT3 (pSTAT3) in control mice, which was exacerbated in the combined presence of GH and CCL4. In STAT5-LKO mice, strong STAT3 activation was observed even before GH injection and was even more pronounced upon GH treatment. Combined CCL4 and GH treatment further enhanced STAT3 activation in STAT5-LKO mice (Fig. 1 E). STAT3 levels were similar in the different groups. Expression levels of the STAT3 target genes fibrinogen γ and haptoglobin were also increased in liver tissue from STAT5-LKO mice and further elevated upon GH and CCL4 treatment (Fig. 1 F and Fig. S3). STAT1 activation was largely independent of CCL4 treatment (unpublished data). These results demonstrate that in the absence of STAT5, GH can directly activate STAT3, and that this activation is strongly enhanced by CCL4.

Development of liver cancer in STAT5-LKO mice

4 out of 20 STAT5-LKO mice had developed multiple tumors after 8 wk of CCL4 treatment, whereas none were detected in 26 control mice (P = 0.0199; Fig. 2 A). Histological analysis revealed that these tumors were mostly formed by moderately differentiated HCCs (Fig. 2 B). It has been established that most HCCs develop as a result of severe liver fibrosis. However, because STAT5-LKO mice had not developed severe fibrosis, we speculated these HCCs were induced through a different mechanism. We again confirmed an almost complete absence of nuclear STAT5B in STAT5-LKO mice, whereas it was present in hepatocytes from control mice (Fig. 2 C). Although little nuclear pSTAT3 was detected in control mice, STAT5-LKO mice exhibited slightly stronger staining of pSTAT3 in non-tumor tissue and extensive staining in tumor tissue (Fig. 2 D). These results indicate that STAT3 is gradually activated in the fibrosis-cancer process in STAT5-LKO mice.

TGF-β protein levels, but not messenger RNA (mRNA) levels, are elevated in STAT5-null mouse embryo fibroblasts and primary hepatocytes

To define the molecular link between STAT5 silencing, STAT3 activation, and elevated TGF-β levels, we performed experiments with mouse embryonic fibroblasts (MEFs) from control and STAT5-null mice. We first confirmed the absence of STAT5 expression and activation in cell lysates from STAT5-null MEFs.
Figure 1. Progression of liver fibrosis in hepatocyte-specific Stat5A/B KO mice. (A and B) Silver staining (A) and Azan staining (B) of liver sections from STAT5A/B^f/f (control) mice and STAT5A/B^f/f;Alb-Cre (STAT5-LKO) mice before and after CCl4 treatment for 8 wk. Arrows indicate reticular fibers. All pictures are shown in low magnification (100×). Bars, 200 μm. (C) Quantification of total acid pepsin-soluble collagens in liver tissue of STAT5A/B^f/f and STAT5A/B^f/f;Alb-Cre before and after CCl4 treatment. Data are mean ± SD. The asterisk indicates a significant difference (P = 0.023) between the control and KO mice (n = 5–7). (D) Expression levels of STAT5A, STAT5b, TGF-β, and LTBP in liver from control and KO mice before and after the treatment as detected by Western blot analysis. (E) Activation levels of STAT5 and STAT3 and expression levels of STAT5, STAT3, and GH receptor (GHR) in liver from control and KO mice before and after CCl4 treatment with or without GH stimulation as detected by Western blot analysis. (F) Expression levels of fibrinogen γ and Haptoglobin in liver from control and STAT5-LKO mice before and after CCl4 treatment with or without GH stimulation as detected by real-time RT-PCR analysis. The relative expression level of the untreated sample of control mice was set as 1, and the fold expression level of each sample was calculated (n = 3 in each group). Data are mean ± SD. Three independent experiments were performed in triplicate and representative data were shown.
by Western blot analyses (Fig. 3 A). Proliferation of control and STAT5-null MEFs was similar both in 15 and 3% FCS (Fig. 3 B). Fibroblastic cells are known to produce TGF-β and to be stimulated by autocrine and paracrine pathways (26). Based on our experiments with hepatocytes, we hypothesized that TGF-β levels would be elevated in STAT5-null MEFs, which
would lead to a paracrine activation and excess production of extracellular matrix protein. The intracellular content of collagen was similar in MEFs from STAT5-null and control mice immediately after their preparation (Fig. 3 C, left). In contrast, elevated collagen levels were observed in STAT5-null but not control MEFs after 10 passages. Similarly, collagen levels were higher in culture medium obtained from STAT5-null MEFs after 1 mo in culture (Fig. 3 C, right). Furthermore, collagen levels were reduced in MEFs of the 10th passage in the presence of anti-TGF-β antibody, suggesting that increased levels of TGF-β caused excess production of collagen. TGF-β levels were greatly elevated in cell lysates and medium of STAT5-null MEFs compared with control MEFs (Fig. 3 D, top; and Fig. S4 ). GH-induced STAT3 activation was also elevated in STAT5-KO MEFs (Fig. 3 D, bottom), which is similar to what was observed in liver section from treated mice. Increased TGF-β levels and GH-induced STAT3 activation were also observed in primary hepatocytes stimulated by CCl₄ (Fig. S5).

To confirm that excessive collagen production was linked to an increase of TGF-β, protein and mRNA levels in cell lysates were examined. Mature TGF-β levels were ~20-fold elevated in STAT5-null MEFs, and these differences were independent of IL-6 or TGF-β stimulation (Fig. 3 E). However, mRNA levels for TGF-β were similar in both cells irrespective of IL-6 or TGF-β stimulation (Fig. 3 F).

**Loss of STAT5 enhances the stability of mature TGF-β**

Because TGF-β mRNA levels were similar in control and STAT5-null MEFs, we examined the effects of STAT5 on TGF-β protein stability and monitored TGF-β levels after inhibiting protein synthesis with cycloheximide (CHx). Serum-starved wild-type and STAT5-null MEFs were treated with CHx for 0–6 h, harvested, and protein was extracted. Although the levels of mature TGF-β remained fairly stable in STAT5-null cells, they rapidly declined in control cells. Quantitative analysis confirmed an elevated half-life of mature TGF-β in STAT5-null MEFs (Fig. 3 G). To understand the underlying mechanism, we tested the effects of the lysosomal inhibitor ammonium chloride on the loss of mature TGF-β. Pretreatment with this inhibitor restored the levels of mature TGF-β in wild-type MEFs in the presence and absence of CHx, whereas it did not change levels in STAT5-null MEFs (Fig. 3 H). These results demonstrate that loss of STAT5 enhances the stability of mature TGF-β protein.

**Interaction of the STAT5 N terminus with TGF-β**

We next used a combination of immunoprecipitation (IP) and immunoblotting to explore the possibility of a physical interaction between STAT5 and TGF-β (Fig. 4, A and B). Cell lysates from STAT5-null and control MEFs, as well as a mixture of the two lysates, were applied to IP followed by immunoblotting. TGF-β was found to interact with STAT5 in extracts from wild-type cells (Fig. 4 A, top). STAT5-null cells served as a negative control. Binding of TGF-β to STAT5B was detected in mixed lysates (Fig. 4 A, bottom). To confirm the interaction between TGF-β and STAT5, we performed the reverse assay performing the IP with antibody against TGF-β (Fig. 4 B). Again, binding of STAT5A and STAT5B to TGF-β was established.

Mature TGF-β binds to LTBP, forming latent TGF-β, which is subsequently secreted. We examined which part of the TGF-β complex binds to STAT5 (Fig. 4 C). STAT5A/B were not detected in immunoprecipitates using LTBP antibodies and LTBP was also not identified in immunoprecipitates using STAT5A/B antibodies. TGF-β was found to bind to STAT5A/B but not to LTBP. Furthermore, these results indicate that the TGF-β complex is not detected inside cells after it binds to LTBP, whereas TGF-β is present in the cytoplasm after it binds to STAT5A/B.

To determine whether known functional amino acids in STAT5 are required for the interaction with TGF-β, we generated retroviral vectors encoding wild-type or mutant STAT5. First we prepared retroviruses carrying the wild-type STAT5a gene as well as mutations in amino acids, which are crucial for the function of STAT5A (27–29). None of these mutations adversely affected the interaction between STAT5A and TGF-β, and their presence led to the suppression of TGF-β levels in STAT5-null MEFs, whereas mRNA levels for TGF-β were similar in these cells (Fig. 4 D and Fig. S6).

To further determine which region of STAT5 interacts with TGF-β, we generated expression vectors carrying either wild-type STAT5 (pWild-T7) or various deletion mutants (pΔC1-T7, pΔC2-T7, pΔN1-T7, and pΔN2-T7; Fig. 4 E). Using an in vitro transcription–translation system with l-[35S]methionine, similar levels of wild-type and mutant STAT5A proteins were synthesized (Fig. 4 F). Subsequently, these STAT5 proteins were incubated with cellular lysates from STAT5-null MEFs and immunoprecipitated with antibody against TGF-β (Fig. 4 G). STAT5AAC1 and ΔC2, as well as wild-type STAT5A, were detected after SDS-PAGE fractionation, whereas STAT5AΔN1 and ΔN2 did not bind to TGF-β. We conclude that the 94 N-terminal amino acids of STAT5 are critical for the interaction with TGF-β.

Because STAT5a and STAT3 share similarities with STAT5 (30), we asked whether their N termini were also able to interact with TGF-β. Using in vitro expression vectors encoding the N-terminal domains of STAT5A/B, STAT3, and STAT1 we determined that the N-terminal domains of STAT5A/B, but not those of STAT3 and STAT1, interacted with TGF-β (Fig. 4, H–J).

To determine the intracellular localization of each protein, we generated vectors expressing TGF-β with a V5 tag and STAT5A coexpressing GFP. STAT5-null MEFs were infected with the STAT5A-expressing retrovirus and subsequently transfected with the TGF-β–V5 plasmid. GFP, an indicator of STAT5A expression, was found preferentially in the nucleus but also in the cytoplasm (Fig. 4 K, top right). In contrast, TGF-β was detected preferentially in the cytoplasm (Fig. 4 K, top left). Cytoplasmic colocalization of STAT5A and TGF-β was observed (Fig. 4 K, bottom right).
Figure 3.  TGF-β protein levels are enhanced in STAT5A/B-null MEFs. (A) Changes in the pSTAT5 and STAT5B levels in control and STAT5-null MEFs before and after GH stimulation as detected by Western blot analysis. (B) Control and STAT5-null MEFs were seeded on a 96-well culture plate, maintained in 15% FCS (left) or 3% FCS (right), and viable cells were assessed by the WST colorimetric assay. Data are mean ± SD. (C) Quantification of total collagen in cell lysates of MEFs (left) or in culture medium (right). Primary MEFs (bars 1 and 4) or MEFs after 10 passages in the absence (bars 2 and 5) or presence (bars 3 and 6) of TGF-β antibody were used. Neutralization antibody was added to the medium every passage (final concentration, 100 ng/ml).
**TGF-β levels are controlled by STAT5**

We have demonstrated an interaction between STAT5 and TGF-β and that elevated TGF-β levels in STAT5-null cells are a result of an enhanced stability. To further establish a causal relationship between STAT5 and TGF-β levels, we expressed wild-type STAT5 and different mutants in STAT5-null MEFs and analyzed TGF-β levels. The efficiency of retroviral infection was equivalent for all constructs (Fig. 4 L). GFP-positive cells were isolated, and subjected to Western blot analysis. TGF-β levels were elevated in STAT5-null MEFs compared with wild-type cells. Infection with retroviruses expressing wild-type, ΔC1, and ΔC2 STAT5A decreased TGF-β to almost undetectable levels. In contrast, TGF-β levels remained unaltered upon expression of STAT5ΔN1 and ΔN2 (Fig. 4 M).

To address the question of whether the interaction with STAT5 would prevent TGF-β from shuttling to lysosomes, we purified lysosomes and analyzed the presence of STAT5-TGF-β complexes. This complex was present in lysosomes in a STAT5A-STAT5B manner (Fig. S7), suggesting that binding to STAT5 does not prevent TGF-β shuttling to lysosomes.

**GH-induced STAT3 and STAT5 activation is modulated by TGF-β**

To explore the physiological consequences of the STAT5–TGF-β interactions on STAT5 activity, we infected control MEFs with a retrovirus expressing TGF-β/GFP. After treatment with or without GH, GFP-positive cells were isolated and protein extracts were analyzed. Phosphorylated STAT5 was detected upon GH stimulation in the absence of exogenous TGF-β but not in its presence (Fig. 5). Intriguingly, strong GH-induced STAT3 activation was detected only in the presence of exogenous TGF-β. The levels of STAT5A/B, STAT3, and GH receptor were equivalent before and after GH stimulation irrespective of TGF-β expression. These findings demonstrate that TGF-β levels determine whether GH activates STAT3 or STAT5.

**The STAT5 N terminus is critical for the development of liver fibrosis**

To further explore the in vivo role of the STAT5 N-terminal domain in the process of liver fibrosis, we took advantage of mice that carry one allele encoding STAT5A/B and the other allele encoding a N-terminal truncated form of STAT5 (31). We generated mice that carry one allele encoding STAT5A/B lacking the first 90 amino acids, one Stat5 floxed allele, and the Alb–Cre transgene (STAT5ΔN;Alb-Cre mice). Western blot analysis confirmed the presence of STAT5A/B-ΔN in liver tissue (unpublished data). Control mice (STAT51/1; STAT51/1;Alb-Cre (STAT5-LKO), and STAT51/ΔN;Alb-Cre (STAT5–ΔN) mice were treated for 8 wk with CCl4. Hematoxylin–eosin (H&E) staining revealed an accumulation of fat droplets in STAT5-LKO but not in control or STAT5–ΔN mice (Fig. 6 A, top). Upon treatment with CCl4, we observed extended reticular fibers that developed similarly in STAT5–ΔN and STAT5-LKO mice but to a lesser extent in controls (Fig. 6 A, middle). Azan staining revealed the formation of nodules and collagen accumulation in STAT5–ΔN and STAT5-LKO mice (Fig. 6 A, bottom). In agreement with these histological findings, increased levels of hepatic collagen were detected after CCl4 treatment (unpublished data). However, no tumors were detected in 17 STAT5–ΔN mice. These results indicate that the STAT5 N terminus is dispensable for the tumor suppressor function of STAT5. Furthermore, it strongly suggests that fat accumulation in STAT5-LKO mice has almost no effect on the fibrotic process because STAT5–ΔN and STAT5-LKO mice had a similar degree of liver fibrosis after treatment.

**DISCUSSION**

GH resistance and low serum levels of insulin-like growth factor 1 have been identified in a large percentage of cirrhotic patients (23, 32), suggesting a link between STAT5 signaling and liver fibrosis and possibly HCC. In this paper, we have demonstrated that TGF-β directly interacts with STAT5 and that elevated levels of TGF-β abrogate GH-induced STAT5 activation. Instead, elevated levels of TGF-β result in GH activation of STAT3, and constitutive STAT3 activity is commonly observed in HCC. Elevated phosphorylation of STAT3 has been associated with epithelial cancers and linked to the induction of STAT3 target genes. In support of a STAT5–TGF-β–STAT3 mechanism, deletion of STAT5 from hepatocytes in mice resulted in elevated levels of TGF-β, an activation of STAT3, and, finally, the development of HCC. We hypothesize that a TGF-β–mediated abrogation of STAT5 signaling results in the activation of STAT3, which is a key step in the development of HCC (Fig. 6 B).

Most likely, the development of liver cancer in this model is the result of more than one molecular insult. Notably, excessive STAT3 activation upon GH and CCl4 treatment in STAT5-LKO mice could result in unscheduled cell proliferation and transformation. TGF-β binds to the N terminus of STAT5, and
Figure 4. STAT5 binds to TGF-β and controls its levels. (A and B) Cellular lysates from wild-type and/or STAT5-null MEFs were immunoprecipitated with nonspecific γ-globulin and antibodies against STAT5A/B or TGF-β, and the immunoprecipitates were subjected to Western blotting. (C) Cellular lysates from STAT5 wild-type MEFs were immunoprecipitated with nonspecific γ-globulin and antibodies against LTBP or STAT5A/B. The immunoprecipitates were subjected to Western blotting. (D) Expression levels of STAT5A and TGF-β in wild-type MEFs, STAT5-null MEFs, and STAT5-null MEFs infected with various kinds of retroviral STAT5-expressing vector. After infection, GFP-negative/positive cells were sorted and applied to Western blot analysis. (E and H) Schematic presentation of protein-expressing plasmids. (F and I) Aliquots of in vitro translation products from each plasmid were fractionated with various kinds of retroviral STAT5-expressing vector. After infection, GFP-negative/positive cells were sorted and applied to Western blot analysis. (G) Cellular lysates from STAT5 wild-type MEFs were immunoprecipitated with nonspecific γ-globulin and antibodies against TGF-β, STAT5A, STAT5B, STAT3, and GH receptor in STAT5 wild-type MEFs before and after GH stimulation were determined by Western blot analysis. STAT5 wild-type MEFs were infected with a retrovirus based on pMSCV–TGF-β–V5, which expresses a TGF-β–V5 fusion protein. Cells were analyzed for TGF-β and STAT5A using γ-globulin and antibodies against STAT5A/B or TGF-β. The immunoprecipitates were subjected to Western blotting. (C) Cellular lysates were subjected to Western blotting. (D) Expression levels of STAT5A and TGF-β in wild-type MEFs, STAT5-null MEFs, and STAT5-null MEFs infected with various kinds of retroviral STAT5-expressing vector. After infection, GFP-negative/positive cells were sorted and applied to Western blot analysis.

Figure 5. GH–induced STAT3 and STAT5 activation is modulated by TGF-β. Phosphorylation levels of STAT5 and STAT3 and expression levels of TGF-β, STAT5A, STAT5B, STAT3, and GH receptor in STAT5 wild-type MEFs before and after GH stimulation were determined by Western blot analysis. STAT5 wild-type MEFs were infected with a retrovirus based on pMSCV–TGF-β–GFP vector. Nuclei were stained with DAPI (blue). Bar, 10 µm. (L) Flow cytometry analysis of STAT5-GFP–infected cells from STAT5 KO MEFs infected with various kinds of STAT5-expressing retrovirus. After infection, GFP-positive cells were sorted and applied to Western blot analysis.
Figure 6. The N-terminal domain of STAT5 is critical for the development of liver fibrosis. (A) H&E staining of liver sections from control, STAT5-LKO, and STAT5-ΔN mice before CCl4 treatment (top) and silver (middle) and azan (bottom) staining of liver sections from these mice after CCl4 treatment. Arrows indicate reticular fibers. H&E images are shown at high magnification (400×; bars, 50 µm), and others are at low magnification (100×; bars, 200 µm). n = 3–5 in each group. (B) Model for the development of liver fibrosis and cancer through elevated expression of TGF-β and activation of STAT3 in hepatocytes. Under physiological conditions, TGF-β is present at low or nondetectable levels and some is secreted after binding to LTBP in a constitutive fashion. STAT5 binds to TGF-β and interferes with the formation of TGF-β complex, resulting in suppression of TGF-β protein stability. At this level of TGF-β, STAT5 levels and activation are not influenced. Upon development of liver fibrosis, TGF-β levels increase and LTBP levels decrease. Under these conditions, STAT5 is sequestered by TGF-β and can no longer be activated by GH. Instead, GH activates STAT3.
of promoters harboring glucocorticoid response elements. As for HNF-4α, it strongly inhibits GH-induced STAT5B transcriptional activity. Although STAT5 decreases TGF-β stability, resulting in a reduction of TGF-β, excess levels of TGF-β inhibit GH-induced activation of STAT5. In this context, GH preferentially activates STAT3, which in turn activates genes linked to cell proliferation and survival. Thus, STAT5 transcriptional functionality can be modulated by activating and suppressing cofactors (50).

The unscheduled ability of STAT3 to be activated by cytokines that normally only activate STAT5 is no artifact of the STAT5-KO mouse model. Recently, several inactivating STAT5B mutations have been identified in humans (51, 52) and linked to dwarfism and immunological disorders. Stimulation of primary cells from these patients with GH and prolactin leads to an unscheduled activation of STAT3 (52), and it is conceivable that some of the immunological disorders observed in these patients can be linked to the aberrant activation of STAT3 rather than the loss of STAT5B. With respect to liver fibrosis and HCC, we propose that TGF-β–mediated down-regulation of STAT5 levels in hepatocytes leads to an aberrant activation of STAT3.

MATERIALS AND METHODS

Mice and treatment. Through breeding, we generated STAT5A/β+/−/α−/−, STAT5A/α−/−, and STAT5A/β+/−/α−/− mice, which are in a mixed background (STAT5−/− mice were provided by J.N. Ihle, St. Jude’s Children’s Research Hospital, Memphis, TN (30, 33, 53). Only 10–18-wk-old female mice were used in the experiment unless otherwise indicated. Primers for genotyping STAT5 was described elsewhere (33). Hepatic fibrosis in mice was induced by i.p. injection with 2 ml/kg body weight of 10% CCl4. Phospho-STAT5 (Tyr694), phospho-STAT3 (Tyr705), and TGF-β antibodies were purchased from Santa Cruz Biotechnology, Inc. The rabbit polyclonal anti-STAT5A (L-20), anti-STAT5B (C-17), and anti-STAT3 (C-20) antibodies were obtained from R&D Systems, and anti-GFP and anti-V5 proteins, we generated two expressing plasmids with a certain tag. One of them is pUB6–TGF-β–V5, which carries the entire TGF-β gene attached with V5 tag in the C terminus of TGF-β, and the other is pmCSV–GFP–STAT5A, which has the STAT5a gene with GFP in the N terminus of STAT5a.

Collagen quantitation. Collagen concentration in liver was determined using the Sircol collagen assay (Biodye Science). In brief, 50 mg of liver tissue was homogenized, and total acid papain-soluble collagens were extracted overnight using 5 mg/ml papain in 500 ml of 0.5 M of acetic acid. 1 ml of Sircol dye reagent was added to 100 µl of each sample in duplicate at 25°C for 30 min. After centrifugation, the pellet was suspended in 1 ml of alkali for 30 min. After centrifugation, the supernatant was used for Western blot to detect the TGF-β or STAT5A/B antibody, or with the rabbit nonspecific γ-globulin (Dako) for 1 h. The immune complex was eluted by being boiled for 3 min. Finally, the supernatant was used for Western blot to detect the TGF-β or STAT5A/B. To confirm the binding site of STAT5A protein to TGF-β protein, several STAT5A proteins possessing the wild-type or some deleted amino acid sequences were synthesized from pΔC1–T7, pΔC2–T7, pΔN1–T7, pΔN2–T7 (indicated in Fig. 4 E), pSSAΔc, pSSBΔc, pSSΔc, and pSΔc (indicated in Fig. 4 H) by the in vitro translation method. The reaction was performed with the TNT T7 coupled reticulocyte lysate system (Promega) and labeled with l-[35S]methionine (GE Healthcare). The cellular lysates from STAT5 wild-type MEFS were extracted and mixed with various types of the in vitro–synthesized STAT5A/B, STAT3, or STAT1 proteins. The mixture was then subjected to IP using antibody against TGF-β as described in the first half of this paragraph. Finally, the immunoprecipitate was separated with SDS-PAGE and the gel was dried and autoradiographed.

Plasmid constructs. Retroviral-expressing vectors of wild-type STAT5A, partially deleted STAT5A, and TGF-β were constructed from pMSCV-IRES GFP new polyn linker (provided by R. Moriggl, Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria). First, pMSCV-STAT5A-GFP or pMSCV–TGF-β–GFP, carrying the entire STAT5a complementary DNA (cDNA) or TGF-β cDNA, was made up by inserting each gene into EcoRI and SacII sites. Four kinds of single amino acid–mutated STAT5a expression vectors were generated by site-directed mutagenesis (T92A, from aca to gca; T97A, from aca to gcc; Y694F, from tac to tcc; and S725A, from tcc to gcc). Other retroviral-expressing vectors of partly deleted STAT5a, such as ΔC1, ΔC2, ΔN1, and ΔN2, were also made up by inserting each PCR product (indicated in Fig. 4 E). For the synthesis of entire or partly deleted STAT5 protein in vitro, we also made five kinds of expressing plasmids, each of which carries the entire or deleted region of STAT5a downstream of the T7 promoter based on pET161/GW/D-TOPO (Invitrogen). To examine the intracellular localization of STAT5 and TGF-β proteins, we generated two expressing plasmids with a certain tag. One of them is pUB6–TGF-β–V5, which carries the entire TGF-β gene attached with V5 tag in the C terminus of TGF-β, and the other is pmCSV–GFP–STAT5A, which has the STAT5a gene with GFP in the N terminus of STAT5a.

Collagen quantitation. Collagen concentration in liver was determined using the Sircol collagen assay (Biodye Science). In brief, 50 mg of liver tissue was homogenized, and total acid papain-soluble collagens were extracted overnight using 5 mg/ml papain in 500 ml of 0.5 M of acetic acid. 1 ml of Sircol dye reagent was added to 100 µl of each sample in duplicate at 25°C for 30 min. After centrifugation, the pellet was suspended in 1 ml of alkali reagent, and absorbance was read at 540 nm.

Production and infection of retrovirus. For making retrovirus, 293T cells were transfected with various kinds of pMSCV-IRES-GFP and EcoPac (the plasmid containing packaging sequences, pkI6.1MCV.ecopac.UTd) using FuGENE (Roche). All supernatants were collected for 48–72 h after transfection and passed through a 0.45-µm filter before freezing at −80°C.

For the infection of retrovirus to MEFs, 10^5 cells were seeded on a 10-cm culture dish and were infected the next day with retrovirus in the presence of 8 µg/ml polybrene. 48–72 h after infection, the cells were lysed and subjected to SDS-PAGE. The cellular protein was extracted from control and STAT5−/− MEFs and preclarified by incubation with protein A-Septharose beads at 4°C for 1 h. Next, the sample was incubated with the beads coupled to the TGF-β or STAT5A/B antibody, or with the rabbit nonspecific γ-globulin (Dako) for 18 h. The immune complex was eluted by being boiled for 3 min. Finally, the supernatant was used for Western blot to detect the TGF-β or STAT5A/B. To confirm the binding site of STAT5A protein to TGF-β protein, several STAT5A proteins possessing the wild-type or some deleted amino acid sequences were synthesized from pΔC1–T7, pΔC2–T7, pΔN1–T7, pΔN2–T7 (indicated in Fig. 4 E), pSSAΔc, pSSBΔc, pSSΔc, and pSΔc (indicated in Fig. 4 H) by the in vitro translation method. The reaction was performed with the TNT T7 coupled reticulocyte lysate system (Promega) and labeled with l-[35S]methionine (GE Healthcare). The cellular lysates from STAT5 wild-type MEFS were extracted and mixed with various types of the in vitro–synthesized STAT5A/B, STAT3, or STAT1 proteins. The mixture was then subjected to IP using antibody against TGF-β as described in the first half of this paragraph. Finally, the immunoprecipitate was separated with SDS-PAGE and the gel was dried and autoradiographed.
Histology. H&E, silver staining, and Azan staining were performed using standard procedures. In short, liver pieces were rapidly removed and fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. The sections were cut to appropriate thickness followed by each staining.

RNA extraction and real-time PCR analysis. Total RNA was isolated (RNasey mini kit; QIAGEN ), and then 1-µg amounts of RNA were reverse transcribed (SuperScript III First-Strand Synthesis SuperMix; Invitrogen), and cDNAs were amplified using Taqman (for fibrinogen γ and haptoglobin) or SYBR Green master mix and probe (for TGF-β1, β2, and β3; Applied Biosystems). These assays were performed according to the manufacturers’ instructions.

Analysis of cell growth. To examine the cell growth curve, 5 × 10^4 wild-type and STAT5 KO MEFs were seeded on a 96-well culture plate. After 3, 6, and 9 d, the net number of viable cells was assessed colorimetrically using water-soluble tetrazolium (WST; 2-[2-methoxy-4-nitrophenyl]-3-[4-nitropheryl]-5-[2,4-disulfophenyl]-2H-tetrazolium monosodium salt; Roche). This assay is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenase in viable cells. For the assay, 10 µl WST reagent was added to the 100-µl culture medium, followed by incubation at 37°C for 2 h. Then the optical density at 450 nm was measured. The assay was done in quadruplicate, and the values were expressed as the means ± SD.

Degradation of TGF-β after blockade of protein synthesis and effects of lysosome inhibitors on TGF-β stability. STAT5 wild-type (two independent lines) and KO MEFs were grown to 80% confluence in 60-mm dishes and serum starved overnight. Cells were then incubated with 10 µg/ml cycloheximide for 2 h followed by CHXs or PBS treatment. Cells lysates were resolved by SDS-PAGE and blotted with anti-TGF-β antibody.

Statistics. All statistical analyses were performed using a two-tailed unpaired Student’s t test. A p-value of 0.05 or less was considered significant.

Online supplemental material. Fig. S1 shows cell availability in isolated hepatocytes and the ratio of apoptotic cells in liver tissues. Fig. S2 shows expression levels of fibrosarcoma-related gene in liver tissues. Fig. S3 shows expression levels of fibrogin α. Fig. S4 shows serum TGF-β levels in MEF culture medium. Fig. S5 shows phosphorylated STAT3 and TGF-β levels in isolated hepatocytes before and after CCI4 treatment. Fig. S6 shows TGF-β1 expression levels in various kinds of MEFs. Fig. S7 shows the existence of the TGF-β–STAT5 complex in lysosome fraction. Fig. S8 shows the immunological analysis, CD4/CD8 ratios, B cell ratios, cytokine production, and T reg cell ratios. Fig. S9 shows pSmad2 level in isolated HSCs. Fig. S10 shows a large area of some Western blots. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20080003/DC1.

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