Mechanisms of STAT3 activation in the liver of FXR knockout mice

Guodong Li,1,2# Yan Zhu,2,4# Ossama Tawfik,5 Bo Kong,2 Jessica A. Williams,3 Le Zhan,2 Karen M. Kassel,3 James P. Luyendyk,6 Li Wang,7 and Grace L. Guo2,3

1Department of Surgical Oncology, Cancer Treatment Center, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, China; 2Department of Pharmacology and Toxicology, School of Pharmacy, Rutgers University, Piscataway, New Jersey; 3Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas; 4Department of General Surgery, Xuanwu Hospital, Capital Medical University, Beijing, China; 5Department of Pathology and laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas; 6Pathobiology and Diagnostic Inv., Michigan State University, East Lansing, Michigan; and 7Department of Medicine, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, Utah

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In FXR−/− mice, spontaneous liver tumors develop with aging in both genders (10, 35), which is associated with increased inflammation, proliferation, and reduced apoptosis (19, 32–34). FXR deficiency leads to liver tumorigenesis, but mechanisms are not completely understood and may be FXR dependent and/or independent. FXR deficiency causes cholestasis (7, 26), which will lead to liver injury and inflammation (2, 10). It is proposed that inflammation is one of the major mechanisms contributing to liver tumor development in FXR−/− mice, and increased inflammation can lead to hepatic carcinogenesis (3).

One common mechanism by which inflammation leads to liver carcinogenesis is via activating the pathway of IL-6/Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), which serves as a potent promoting factor driving liver tumor formation (36). Activation of STAT3 induces the expression of a cohort of genes that stimulate cell proliferation and reduce apoptosis (36). Remarkably, STAT3 also induces the gene expression of suppressor of cytokine signaling 3 (SOCS3), which feedback suppresses STAT3 activity (5, 27). Emerging evidence has shown that FXR deficiency leads to STAT3 activation; however, the underlying mechanism remains unknown.

The current study determined the molecular basis by which FXR deficiency leads to hepatic STAT3 activation in mice. We demonstrate that both FXR-dependent and -independent mechanisms are involved in activating STAT3. This study will advance our understanding of the molecular mechanisms of liver carcinogenesis under FXR-deficient conditions.

MATERIALS AND METHODS

Animals and treatments. Male wild-type (WT) and FXR−/− mice were on a C57BL/6J genetic background. The mice were housed and bred in pathogen-free animal facilities in the Laboratory of Animal Research under a standard 12:12-h light-dark cycle with free access to food and autoclaved tap water. The protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Mice were killed at 3, 6, 12, and 18 mo of age to determine liver gene expression. For the cholestyramine feeding study, male WT and FXR−/− mice were fed a 2% (wt/wt) cholestyramine-containing diet or regular diet (LabDiet, Olathe, KS) from 3 wk to 3 mo of age. For the cholic acid treatment study, 3- to 4-mo-old male WT and FXR−/− mice were orally gavaged with 75 mg/kg GW-4064 or vehicle two times (first dosage at 6:00 P.M. and second dosage at 8:00 A.M. the next day), with livers

#G. Li and Y. Zhu contributed equally to this work.

Address for reprint requests and other correspondence: G. L. Guo, Dept. of Pharmacology and Toxicology, School of Pharmacy, Rutgers Univ., Piscataway, NJ 08854 (e-mail: guo@ehols.rutgers.edu).

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harvested 4 h after the second treatment. The source of GW-4064 and its preparation was previously described (14).

Serum alanine aminotransferase activity and total BA levels, as well as serum and liver IL-6 levels. Serum alanine aminotransferase (ALT) activity, BA levels, and concentrations of IL-6 in mouse serum and livers were determined using commercially available kits (Pointe Scientific, Canton, MI; Bio-Quant, San Diego, CA; R&D Systems, Minneapolis, MN, respectively), according to the manufacturers’ instructions.

RNA isolation and quantitative real-time PCR. Total RNA isolation, quantification, and quantitative real-time PCR (Q-PCR) were performed according to standard procedures as described previously (14). The concentration of total RNA was determined by spectrophotometry with the integrity confirmed by MOPS gel electrophoresis. The mRNA expression levels of Fxr, Shp, Cyp7a1, Ostβ, Ntcp, Bsep, Fgf15, Il6, IL-1α, IL-1β, c-Myc, Survivin, p21, and Socs3 were quantified by Q-PCR using SYBR green chemistry (Fermentas, Glen Burnie, MD) and normalized to Gapdh mRNA levels. The primers are available upon request.

BA pool size determination. The total BA pool size was determined by measuring BAs of the small intestine, gallbladder, liver, and their contents. All mice were weighed before being killed. Fresh organs were collected and homogenized together. The total BAs in homogenate were extracted according to a method described previously (12). The BA pool size was expressed as micromoles of BAs per 100 g of body weight.

Western blot analysis. Nuclear and cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were measured by a BCA protein

Fig. 1. Activation of signal transducer and activator of transcription 3 (STAT3) in livers of farnesoid X receptor-deficient (FXR−/−) mice. A, left: Western blot analysis of total hepatic protein levels of phosphorylated (p) STAT3, total STAT3, phosphorylated STAT1, and total STAT1 at 3 and 12 mo of age in wild-type (WT) and FXR−/− mice. Each band represents one single mouse sample in the indicated genotype group. A, right: the band density was determined using the Imagej software. *Significant difference between WT and FXR−/− mice. B, left: phosphorylated and total STAT3 from 3-, 6-, 12-, and 18-mo-old WT and FXR−/− mice. Each band represents a pooled sample from 3 individual mice with the same genotype. B, right: band density. C, left: Western blot analysis of STAT3 downstream targets, c-Myc, p21, and suppressor of cytokine signaling 3 (SOCS3), from WT and FXR−/− mice at 3 mo old. Each band represents a pooled sample from 3 individual mice with the same genotype. C, right: band density. β-Actin levels were used as an internal loading control for all blots.
detection kit (Pierce, Rockford, IL). Western blot analysis was performed as previously described on total, nuclear, and cytoplasmic proteins (18). Antibodies information is available upon request. β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

Chromatin immunoprecipitation coupled with high-throughput DNA sequencing and chromatin immunoprecipitation-qPCR. Chromatin immunoprecipitation coupled with high-throughput DNA sequencing (ChIP-seq) and chromatin immunoprecipitation (ChIP)-qPCR were per-

Fig. 2. Decreased bile acid (BA) pool reduced Janus kinase 2 (JAK2)/STAT3 activation. WT and FXR−/− mice were fed a 2% cholestyramine-containing diet for ~10 wk. A: total BA pool size, serum BA levels, alanine aminotransferase (ALT) activity, and serum and liver IL-6 levels. The BA pool size is expressed as micromoles of BAs/100 g of body wt. B: the mRNA levels of FXR target genes and inflammatory cytokines in WT and FXR−/− mice fed the cholestyramine-containing or regular diet. *P < 0.05 and **P < 0.01 between vehicle- and cholestyramine-treated WT mice. #P < 0.05 and ##P < 0.01 between vehicle- and cholestyramine-treated FXR−/− mice. C, left: Western blot analysis of total hepatic protein levels of phosphorylated STAT3, total STAT3, phosphorylated JAK2, and total JAK2 in WT and FXR−/− mice fed with regular or cholestyramine-containing diet. Each band represents a pooled sample from 3 individual mice in the same group. C, right: band density.
formed on livers of mice treated with vehicle or GW-4064 as previously described (29). Histograms of FXR binding to the Soxs3 gene in liver were generated using Affymetrix Integrated Genome Browser, and three FXR binding sites (A, B, and C) were located in the downstream region of the Soxs3 gene. The purified DNA fragments that were bound by FXR were analyzed by Q-PCR, and Ostβ gene was used as a positive control. The FXR−/− mice treated with GW-4064 were used as negative controls. The relative intensity (fold) indicates fold increase over vehicle treatment. The sequences of the primers for ChIP-qPCR assay are available upon request.

Fig. 3. Increased BA levels activate the JAK2/STAT3 signaling pathway. Male WT and FXR−/− mice, 2 to 3 mo old, were fed regular or 1% cholic acid-containing diet for 5 days. A: serum BA levels, ALT activity, and serum IL-6 levels. B: the mRNA levels of FXR target genes and inflammatory cytokines in each group of mice. *P < 0.05 and **P < 0.01 between vehicle- and cholic acid-treated groups of WT mice. #P < 0.05 and ##P < 0.01 between vehicle- and cholic acid-treated groups of FXR−/− mice. C, left: Western blot analysis of total hepatic protein levels of phosphorylated STAT3, total STAT3, phosphorylated JAK2, and total JAK2 in WT and FXR−/− mice treated with vehicle or cholic acid. Each band represents a pooled sample from 3 individual mice in the same group. C, right: band density.
Construction of plasmids for reporter gene luciferase assay, site-directed mutagenesis of FXR response elements (FXRREs), cell culture, transient transfection, and luciferase reporter gene assays. A fragment containing both of the FXRREs in the form of an IR1 in the downstream regulatory region of the Socs3 gene, from +3741 to +6403 relative to the transcription start site, was amplified from mouse genomic DNA by PCR by methods described earlier (15, 16). The sequences of the primers are available upon request. The cloned construct was validated by DNA sequencing, and the new plasmid was named PGL4-Socs3-TK luciferase vector.

The QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate mutations of the two IR1-binding sites in the PGL4-Socs3-TK luciferase vector with primer information available upon request. The desired mutations were verified by DNA

Fig. 4. Socs3 is a direct FXR target gene. A: the mRNA levels of Fxr and its target genes Shp, Cyp7a1, and Ostβ, Socs3, and cytokines IL-1β, IL-1α, and IL-6 by quantitative real-time PCR (Q-PCR) analysis in livers of WT and FXR−/− mice treated with vehicle or a potent FXR ligand, GW-4064. *P < 0.05 and **P < 0.01 between vehicle- and GW-4064-treated groups of WT mice. #P < 0.05 between WT and FXR−/− mice with vehicle treatment. B: Western blot analysis of total hepatic protein levels of SOCS3 in WT and FXR−/− mice treated with vehicle or GW-4064. Three samples were run individually from each group. Band density was presented under the blots. *P < 0.05 between vehicle- and GW-4064-treated groups of WT mice.
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sequence, and the mutated plasmids were named Mu-PGL4-Socs3-TK-1st IR1, Mu-PGL4-Socs3-TK-2nd IR1, and Mu-PGL4-Socs3-TK-(1st + 2nd) IR1 luciferase vector. Transient transfection was performed as previously described in HepG2 cells (15, 16).

Statistical analysis. All data were presented as means ± SD and analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Activation of STAT3 but not STAT1 in FXR−/− mouse livers. Emerging evidence has shown that FXR deficiency leads to increased liver inflammation. To elucidate how inflammation contributes to liver carcinogenesis under FXR-deficient conditions, we determined the status of STAT3 and STAT1 activation in WT and FXR−/− mice with aging. The results showed that STAT3 activation, as revealed by Tyr705 and Ser727 phosphorylation, was increased in FXR−/− mice at 3 mo of age, which was further increased at 12 mo of age, whereas the total STAT3 protein levels remained unchanged (Fig. 1A). Furthermore, STAT3 was activated in an age-dependent manner (Fig. 1B). In addition, we determined the protein levels of classical target genes of STAT3, and the results showed that c-Myc, but not P21 and Socs3, were induced in the liver of FXR−/− mice (Fig. 1C).

Decrease in BA pool reduces STAT3 activation in livers of FXR−/− mice. FXR−/− mice have higher levels of BAs that are known to induce inflammation and liver injury. Therefore, the effects of BAs on STAT3 activation in FXR−/− mouse livers were determined. As expected, the BA pool size and serum BA levels were reduced in WT and FXR−/− mice by treatment with cholestyramine, a BA sequestrant (Fig. 2A). Cholestyramine markedly reduced serum ALT activity and serum and liver IL-6 levels in FXR−/− but not in WT mice (Fig. 2A). The changes in mRNA levels of FXR and its target genes involved in BA homeostasis in the liver and intestine, including Fxr, Shp, Cyp7a1, Ostβ, Fgf15, and Ibabp, confirmed the effectiveness of cholestyramine treatment at the molecular level. Specifically, cholestyramine treatment led to an increase in mRNA levels of Cyp7a1, Ostβ, Ntcp, and Bsep, in the liver, and it led to a decrease of Shp, Fgf15, and Ibabp mRNA levels in the intestine (Fig. 2B and data not shown). Cholestyramine treatment also decreased IL-1α, IL-1β, and IL-6 mRNA levels in livers of FXR−/− but not WT mice (Fig. 2B). Interestingly, cholestyramine did not alter mRNA levels of Socs3, which were decreased in livers of FXR−/− mice compared with those in WT mice (Fig. 2B). Cholestyramine did not alter JAK1/STAT1 nor SOCS3 protein levels (data not shown) but markedly abolished the activation of JAK2/STAT3 in FXR−/− mouse livers (Fig. 2C).

Increased BAs activate JAK2/STAT3 in livers of WT and FXR−/− mice. In contrast to results for cholestyramine treatment, treatment with a primary BA, CA, resulted in increased serum BA levels, ALT activity, and IL-6 levels in WT mice.

**Fig. 5.** FXR regulates Socs3 gene expression by binding to downstream inverted repeats separated by one nucleotide (IR1s) in the Socs3 gene. **A:** three novel FXR binding sites (A, B, and C) in the downstream region of the Socs3 gene identified by chromatin immunoprecipitation coupled with high-throughput DNA sequencing (ChIP-seq) analysis. An IR1 was identified in binding sites A and B. Binding site C contains a direct repeat (DR) 8. **B:** chromatin immunoprecipitation (ChIP)-qPCR assays show binding of FXR to binding sites A and B, but not to site C, of the Socs3 gene in mouse livers after treatment with GW-4064. The relative intensity (fold) indicates fold increase over vehicle treatment; n = 3 mice/group. **P** < 0.05 and **P** < 0.01 between vehicle- and GW-4064-treated WT livers. #P < 0.05 between WT and FXR−/− mice with GW-4064 treatment. **C:** luciferase assays reveal that the two IR1s within the downstream region of the Socs3 gene were functional in enhancing gene transcription upon FXR activation. The DNA constructs containing these two novel FXR-binding sites (IR1s) and the DNA constructs containing mutant corresponding novel IR1s were cloned into a PGL4-TK firefly luciferase vector and transfected into HepG2 cells as indicated in MATERIALS AND METHODS. The cells were then treated with vehicle (0.1% DMSO) or 1 μM GW-4064 for 36 h followed by evaluation of luciferase activity. Open bar, cells treated with vehicle; closed bar, cells treated with 1 μM GW-4064. The relative luciferase activity (fold) indicates fold increase over empty vector by vehicle treatment. **P** < 0.05 between vehicle and GW-4064 treatment.
with further increase in FXR\textsuperscript{−/−} mice (Fig. 3A). Activation of hepatic FXR by CA was verified by changes in mRNA levels of FXR target genes (decrease in Cyp7a1 and increase in Shp and Ostβ) (Fig. 3B). Hepatic mRNA levels of IL-1β and IL-6, but not of IL-1α, were increased by CA treatment in WT mice, and were further increased in FXR\textsuperscript{−/−} mice (Fig. 3B). The same trend was found for STAT3 target genes, Socs3, c-Myc, and p21 (Fig. 3B). CA increased phosphorylation of JAK2 and STAT3 in WT and FXR\textsuperscript{−/−} mice (Fig. 3C). Furthermore, the protein levels of SOCS3 and c-Myc, but not p21, were increased following CA treatment, further confirming activation of the JAK2/STAT3 pathway (data not shown). JAK1/STAT1 protein levels were not changed by CA (data not shown).

SOCS3, the feedback inhibitor of STAT3, is a FXR target gene. As mentioned earlier, SOCS3 inhibits STAT3 activation via a feedback regulation. The reduced basal expression of SOCS3 in FXR\textsuperscript{−/−} mice suggests that SOCS3 may be a FXR target gene. In addition, activation of FXR by GW-4064 induced Socs3 mRNA levels in WT (~3 times), but not in FXR\textsuperscript{−/−}, mice further suggesting FXR-dependent regulation. In contrast, the expression of IL-1α, IL-1β, or IL-6 was not affected by GW-4064 (Fig. 4A). Consistent with changes in mRNA levels, SOCS3 protein levels were also induced by GW-4064 in WT but not in FXR\textsuperscript{−/−} mice (Fig. 4B). These results indicate that Socs3 is likely regulated by FXR, and its basal expression is maintained, at least in part, by FXR in vivo.

Socs3 is a direct FXR target gene. According to the published data of FXR ChIP-seq (4, 29), three regions (A, B, and C) downstream of the Socs3 gene were bound by FXR (Fig. 5A). The first two regions, A and B, contained putative FXRREs in the form of an IR1, and the third binding region, C, was a DR separated by eight nucleotides (Fig. 5A). ChIP-qPCR assays demonstrated that FXR bound to regions A and B but not C in mice treated with vehicle, and FXR bound to all three sites in mice treated with GW-4064, although site C showed a much weaker binding (Fig. 5B). Luciferase reporter gene assays were used to determine whether the two IR1s in the Socs3 gene were functional in enhancing transcriptional activity. Compared with the vehicle control, GW-4064 induced the activity of the luciferase reporter containing two IR1 sites (A and B) in the Socs3 gene by 5.5-fold. This activity was reduced to 3.2-fold by site-directed mutation of site A, and was further reduced to basal level by mutation of site B alone or simultaneous mutation of both sites A and B (Fig. 5C).

**DISCUSSION**

In the present study, we determined mechanisms of STAT3 activation in FXR\textsuperscript{−/−} mice. Increased BAs lead to increased inflammatory cytokines, such as IL-6, to result in STAT3 activation in the liver. Furthermore, SOCS3, the feedback inhibitor of the JAK2/STAT3 pathway, was a direct FXR target gene. Therefore, the abrogation of SOCS3 feedback inhibition further enhanced JAK2/STAT3 activation in FXR\textsuperscript{−/−} mice (Fig. 6). Together, these findings reveal the FXR-dependent and -independent molecular mechanisms to activate STAT3, and these mechanisms may contribute to the liver carcinogenesis in the FXR\textsuperscript{−/−} mice.

We detected the JAK2/STAT3, but not JAK1/STAT1, activation in FXR\textsuperscript{−/−} mouse livers as early as 3 mo of age. This result was in agreement with a recent publication showing similar findings (19). STAT3 activation in FXR\textsuperscript{−/−} mice seems to be mediated through the IL-6-JAK2-STAT3 pathway because the involvement of other critical regulators that activate STAT3, such as NF-κB (8) and mammalian target of rapamycin (11), were not found (data not shown). These results showed that the JAK2/STAT3 pathway is activated by FXR deficiency, which may be a mechanism of liver carcinogenesis in FXR\textsuperscript{−/−} mice. We further showed that a STAT3 target gene, c-Myc, was induced in livers of FXR\textsuperscript{−/−} mice. Studies have already shown that c-Myc was induced in FXR\textsuperscript{−/−} mouse livers, and this induction may be associated with the activation of β-catenin (10). However, our results showed that c-Myc may also be activated through the JAK2/STAT3 pathway in livers of FXR\textsuperscript{−/−} mice, since c-Myc is a downstream target of the JAK2/STAT3 signaling pathway (1).

High levels of BAs have been shown to promote liver tumorigenesis in FXR\textsuperscript{−/−} mice (35). Moreover, we showed that the mRNA levels of the inflammatory genes, IL-1β and IL-6, were induced in livers of FXR\textsuperscript{−/−} mice with aging (data...
not shown). We hypothesize that elevated BAs induce IL-6, which in turn activates STAT3. IL-6 is a potent inflammatory cytokine that plays a critical role in carcinogenesis (20), and its levels were dramatically increased in hepatocellular carcinoma (HCC) patients with higher IL-6 levels associated with primary liver cancer progression (24). We have observed that serum IL-6 levels and liver IL-6 mRNA levels were increased in FXR−/− mice in an age-dependent manner (unpublished data). This increase of IL-6 may be the result of the increased BAs in FXR−/− mice, which activates inflammatory cells, such as Kupffer cells, to produce more proinflammatory cytokines, including IL-6. IL-6 is able to activate the JAK2/STAT3 signaling pathway through binding to glycoprotein 130 (31).

STAT3, which is most commonly activated by IL-6, serves as a crucial regulator of inflammation and carcinogenesis (36). Moreover, STAT3 works as a point of convergence for numerous oncogenic signaling pathways during HCC development. Upon activation, STAT3 induces the transcription of target genes crucial for cell proliferation (31, 36). We have already demonstrated that target genes of STAT3, including c-Myc, P21, and Survivin, were highly induced in livers of aging FXR−/− mice (unpublished data). These results highly suggest that increased BAs and IL-6 likely contribute to activating the JAK2/STAT3 pathway in FXR−/− mice.

The effects of BAs on STAT3 activation are clearly demonstrated by the current study. By manipulating BA levels, we determined the effects of BAs on IL-6 levels and on JAK2/STAT3 activation. Specifically, decreasing the BA pool size reduced serum and liver IL-6 levels while increasing BAs induced serum IL-6 levels, especially in FXR−/− mice. The hepatic expression of IL-1β and IL-6 was also correlated with the change in BA levels. Moreover, the status of STAT3 activation correlated with the changes of IL-6 levels. Increasing evidence indicates that activation of FXR inhibits liver inflammation. Overexpression of constitutively active FXR in mouse livers has been show to inhibit hepatic inflammation (33). Furthermore, the synthetic FXR agonist, WAY-362450, has been shown to protect against the development of nonalcoholic steatohepatitis by reducing liver inflammation and fibrosis in a murine methionine- and choline-deficient model (37). In addition, it has been reported that activation of FXR increases hepatocyte survival by reducing mitochondrial dysfunction and oxidative stress through regulating micro-RNAs (13). Hence, maintaining FXR function in hepatocytes may protect against liver tumorigenesis.

Emerging evidence suggests that blocking STAT3 activation may be a novel strategy for the prevention/treatment of HCC (28). SOCS3 is one of the downstream targets of STAT3 and is a critical feedback inhibitor of the IL-6-STAT3 signaling pathway (5, 27). However, to our surprise, STAT3 activation did not induce SOCS3 expression in livers of FXR−/− mice, further indicating that abrogation of the SOCS3 feedback loop may contribute to the increased activation of STAT3 signaling in FXR−/− mice as well. Further studies revealed that SOCS3 expression was induced in a FXR-dependent manner. In this study, SOCS3 is proven to be a bona fide FXR target gene because FXR bound to two IR1s in the downstream region of the SOCS3 gene and induced gene transcription.

SOCS3 is a target gene of STAT3 and is critical in feedback inhibiting JAK/STAT activation (5). Deletion of the SOCS3 gene results in a prolonged activation of the IL-6-STAT3 signaling pathway (5). SOCS3 expression is reduced in HCC patients, and aberrant hypermethylation in the promoter region of the SOCS3 gene has been demonstrated to contribute to transcriptional silencing of SOCS3 (21). Furthermore, Socs3−/− deficient mice develop HCC at an accelerated rate (22). In addition, restoration of SOCS3 achieved a significant antitumor effect by inhibiting STAT3 activation (6). In fact, Socs3 gene expression was decreased with FXR deficiency, and FXR activation induced SOCS3 expression. Moreover, FXR bound to two IR1s in the downstream gene regulatory region of the Socs3 gene to directly induce gene expression. Thereby, aberrant Socs3 gene expression results in loss of the classical feedback inhibition of STAT3 activation in FXR−/− mice. These results aid in revealing underlying molecular mechanisms of increased activation of STAT3 in FXR−/− mice, which may be, at least partly, responsible for liver carcinogenesis.

Collectively, our results demonstrate that increased activation of the IL-6-STAT3 pathway in the liver by increased BA levels and/or by abrogating the feedback inhibition of STAT3 activation by Socs3 may both contribute to the molecular mechanisms of HCC development in FXR−/− mice. Because the JAK2/STAT3 signaling pathway has been considered as an important factor for HCC initiation, development, and progression, modulation of JAK2/STAT3 pathway activity by FXR may be a promising new therapeutic target for HCC.

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