Dietary and Genetic Obesity Promote Liver Inflammation and Tumorigenesis by Enhancing IL-6 and TNF Expression

Eek Joong Park,[1] Jun Hee Lee,[1] Guann-Yi Yu,[1] Guobin He,[1] Syed Raza Ali,[1] Ryan G. Holzer,[1] Christoph H. Österreicher,[1,2] Hiroyuki Takahashi,[1] and Michael Karin[1,*]
[1]Laboratory of Gene Regulation and Signal Transduction, Departments of Pharmacology and Pathology
[2]Department of Medicine
School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA
*Correspondence: karinoffice@ucsd.edu

SUMMARY

Epidemiological studies indicate that overweight and obesity are associated with increased cancer risk. To study how obesity augments cancer risk and development, we focused on hepatocellular carcinoma (HCC), the common form of liver cancer whose occurrence and progression are the most strongly affected by obesity among all cancers. We now demonstrate that either dietary or genetic obesity is a potent bona fide liver tumor promoter in mice. Obesity-promoted HCC development was dependent on enhanced production of the tumor-promoting cytokines IL-6 and TNF, which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3. The chronic inflammatory response caused by obesity and enhanced production of IL-6 and TNF may also increase the risk of other cancers.

INTRODUCTION

Cancer development and progression are influenced by genetic, epigenetic, and environmental factors. Epidemiological studies are particularly effective for identification of cancer risk factors but rarely elucidate the underlying mechanisms. For instance, a link between inflammation and cancer was first suggested by epidemiological studies, but the underlying mechanisms were revealed through genetic and biochemical studies in appropriate mouse models (Johansson et al., 2008; Karin, 2006; Mantovani et al., 2008). Recently, a number of large-scale epidemiological studies have pointed out that overweight and obesity, defined by body mass index (BMI) higher than 25, result in a substantial increase in cancer risk (Bianchini et al., 2002; Calle and Kaaks, 2004; Calle et al., 2003). It was pointed out that in men and women, respectively, excess body weight results in up to 1.52- or 1.62-fold increases in the relative risk of cancer-related death (Calle et al., 2003). The actual increase in risk is very much dependent on the type of cancer, and the largest increase in cancer risk in individuals with high BMI was seen for hepatocellular carcinoma (HCC), the most common form of liver cancer. Men with BMI of 35–40 exhibited a staggering 4.52-fold increase in relative HCC risk (Calle et al., 2003). Even a modest elevation in BMI to the 25–30 range (grade 1 overweight) resulted in a significant increase in risk of death due to HCC, pancreatic cancer, cancers of the gastrointestinal (GI) tract, and kidney cancer. Given the prevalence of overweight and obesity in the Western world (approximately two-thirds of US adults are overweight or obese; Calle et al., 2003) and their rapid increase in developing countries (there are about 300 million obese individuals worldwide), even a modest 1.5- to 1.6-fold increase in relative cancer risk represents a major public health problem. Indeed, more than 90,000 cancer-related deaths per year in the US alone are linked to excessive body weight (Calle et al., 2003).

Several mechanisms were proposed to explain how obesity increases cancer risk, including the prevalence of type II diabetes and insulin resistance among obese individuals, which result in elevated circulating concentrations of insulin and insulin-like growth factor 1 (IGF-1), as well as increased production of sex steroids and cytokines by adipose tissue (Calle and Kaaks, 2004). However, these proposed mechanisms are based on correlative studies and have not been critically evaluated. Given the large impact of high BMI on HCC risk, we decided to investigate the mechanisms by which obesity promotes HCC development. Furthermore, an association between liver fat accumulation or hepatosteatosis and HCC development has been long known (Caldwell et al., 2004; El-Serag and Rudolph, 2007). Obesity-induced hepatosteatosis, together with its more severe complication nonalcoholic steatohepatitis (NASH), classified as nonalcoholic fatty liver disease (NAFLD), which affects up to 24% of the US population (Parekh and Anania, 2007), may match in their etiologic impact hepatitis C virus (HCV) infection, the major HCC risk factor in the US and Japan (Caldwell et al., 2004; El-Serag and Rudolph, 2007).

HCV infection and NAFLD, which can synergize to cause an even greater increase in HCC risk, can both lead to endoplasmic reticulum (ER) stress and accumulation of reactive oxygen species (ROS) within liver parenchymal cells resulting in chronic liver damage (Li et al., 2009; Parekh and Anania, 2007). It is thought that chronic liver injury that results in compensatory

proliferation of differentiated hepatocytes, which are otherwise quiescent, is one of the major pathogenic mechanisms underlying HCC development (Bisgaard and Thorgeirsson, 1996; Fausto, 1999; Sakurai et al., 2006). The relevance of compensatory proliferation as a tumorigenic mechanism is supported by mouse genetic studies, using either the hepatic procarcinogen diethylnitrosamine (DEN) as an inducer of HCC (Hui et al., 2007; Maeda et al., 2005; Sakurai et al., 2006, 2008) or the conditional and complete inactivation of NF-κB signaling needed for maintenance of hepatocyte survival (Luedde et al., 2007).

We have used the DEN model to investigate whether dietary or genetic obesity is a direct promoter of HCC development and if so dissect its mechanism of action. We now show that obesity is a bona fide liver tumor promoter under conditions where DEN administration to lean mice does not result in HCC induction. We also demonstrate that one of the mechanisms that accounts for the tumor-promoting effect of obesity is the low-grade inflammatory response it induces (Hotamisligil, 2006; Shoelson et al., 2007), which results in elevated production of cytokines, such as TNF and IL-6, which was found to positively correlate with progression of chronic viral hepatitis to HCC in humans (Nakagawa et al., 2009; Wong et al., 2009). These cytokines are also required for propagation of steatohepatitis.

RESULTS

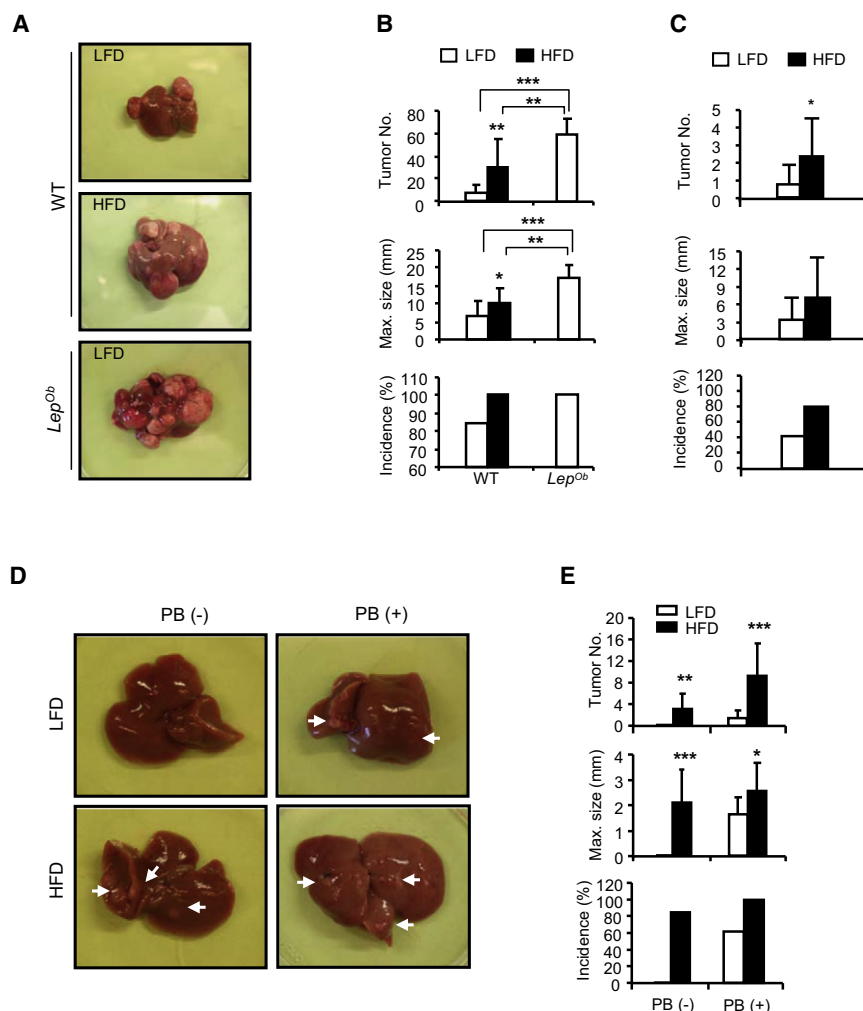### Genetic and Dietary Obesity Strongly Promote DEN-Induced Hepatocarcinogenesis

Although obesity is a well-established major HCC risk factor (El-Serag and Rudolph, 2007), critical mechanistic insights to its tumor-promoting activity are currently lacking. To be able to employ genetic tools in our mechanistic analysis, we decided to conduct our work with mice rather than other rodents. Previous studies had revealed that hepatocyte-specific deletion of the IKK regulatory subunit NEMO/IKKγ results in spontaneous liver damage, hepatosteatosis, liver fibrosis, and HCC development (Luedde et al., 2007). When placed on high-fat diet (HFD), $Nemo/Ikk\gamma^{\Delta hep}$ mice exhibit the same protection from obesity-induced insulin resistance previously found in liver-specific IKKβ knockout ($Ikk\beta^{\Delta hep}$) mice (Arkan et al., 2005) but display enhanced hepatosteatosis and elevated liver damage and eventually develop four times more HCCs than $Nemo/Ikk\gamma^{\Delta hep}$ mice kept on normal chow (Wunderlich et al., 2008). However, due to spontaneous liver damage and hepatosteatosis in $Nemo/Ikk\gamma^{\Delta hep}$ mice, it is difficult to determine whether obesity alone is a tumor promoter in this case. To address this issue more definitively, we decided to use wild-type (WT) C57/BL6 mice which do not develop spontaneous liver disease or cancer but are susceptible to the hepatic procarcinogen DEN, which effectively induces HCC formation when given to 14-day-old mice (Vesselinovitch and Mihailovich, 1983).

We administered DEN (25 mg/kg) to either male or female mice on postnatal day 14 and 4 weeks later placed the mice on either normal chow (LFD) or high-fat diet (HFD), in which 60% of calories are fat derived (protocol #1, Figure S1A). As expected, male mice maintained on HFD gained more weight than mice on LFD (Figure S1C) and their relative liver weight was increased (Figure S1D), as were liver triglycerides (TG) (Figure S1E). This

was accompanied by increased serum TG (Figure S1F) and hepatosteatosis (Figure S1G). As expected, mice on HFD became glucose intolerant (Figure S1H). Importantly, when analyzed at 9 months of age, mice kept on HFD exhibited many more HCCs per liver than LFD-maintained mice (Figures 1A and 1B). Dietary obesity also increased tumor size and incidence. A similar effect was seen in female mice that were subjected to the same HCC induction protocol (Figure 1C). Due to their lower susceptibility to DEN-induced carcinogenesis (Naugler et al., 2007), female mice exhibited a greater enhancement of HCC incidence than male mice, although tumor multiplicity and its enhancement by obesity were lower in females. HFD increased body and liver weight as well as serum and liver TG also in female mice (Figures S1I–S1K), but the effects on liver weight and TG content were smaller than those seen in males.

To rule out the contribution of any potential carcinogens or tumor promoters that could be present in HFD but not in normal chow, we examined whether genetic obesity also enhances HCC induction and development. As leptin-deficient mice (Lep^{Ob}) spontaneously develop obesity even when kept on normal chow (Friedman et al., 1991; Pelleymounter et al., 1995), we used them as a model for genetic obesity. We administrated 25 mg/kg DEN to 2-week-old Lep^{Ob} male mice and found that they exhibited greatly enhanced HCC development relative to WT mice kept on LFD (Figures 1A and 1B). These results strongly suggest that it is either obesity per se or hepatosteatosis that enhances HCC induction and development rather than presence of unknown carcinogens or tumor promoters in the particular HFD that we used.

Next, using conditions under which lean WT mice do not develop any liver tumors when given DEN, we examined whether dietary obesity functions as a tumor promoter. We took advantage of the earlier observation that when DEN administration is delayed until 4–6 weeks of age, it fails to induce HCC, even in males, unless combined with a tumor promoter, such as phenobarbital (PB) (Ward et al., 1986). We kept WT male mice on either LFD or HFD for 10 weeks prior to DEN administration at 16 weeks of age (protocol #2, Figure S1B). As expected, HFD consumption initially resulted in weight gain and led to elevated serum TG (Figures S2A and S2B). However, immediately after DEN administration, HFD-kept mice lost weight but regained it a few weeks later. This sudden weight loss is likely to be due to enhanced DEN-induced liver toxicity in obese mice, first noted when HFD-kept mice were given the standard carcinogenic dose of 100 mg/kg DEN, which is not toxic in lean mice. Under these conditions, all of the obese mice died within 13 days after DEN administration (Figure S2C). Death was likely to have been caused by liver failure as indicated by histological analysis (Figure S2D), TUNEL staining (Figure S2E) and massive release of the liver enzyme alanine aminotransferase (ALT) into the circulation (Figure S2F). Obese mice also exhibited enhanced DEN-induced ROS accumulation (Figure S2G). We therefore reduced the dose of DEN used in this protocol (protocol #2, Figure S1B) to 80 mg/kg. At this dose, despite initial weight loss (Figure S2A), no lethality was observed in the HFD group. Remarkably, close to 90% of mice kept on HFD developed HCC without PB administration (Figures 1D and 1E). By contrast, and as expected, mice kept on LFD did not develop any HCC

Figure 1. Genetic and Dietary Obesity Promote DEN-Induced Hepatocarcinogenesis

(A) Livers of male WT and Lep^{Ob} mice kept on normal chow (LFD) or high-fat diet (HFD) from week 6 to week 36 after the administration of DEN (25 mg/kg) at 2 weeks of age (protocol #1; Figure S1A).

(B) Tumor multiplicity, size, and incidence in livers of DEN-injected WT and Lep^{Ob} male mice kept on LFD (□) or HFD (■) as above. Results are averages ± standard deviation (SD) (n = 10–12).

(C) Tumor multiplicity, size, and incidence in livers of WT females that were given DEN at 2 weeks of age and fed as above. Results are averages ± SD (n = 10–12).

(D) Livers of WT male mice given DEN (80 mg/kg) at 16 weeks of age and kept on LFD or HFD from week 6 to week 50 (protocol #2; Figure S1B). When indicated, phenobarbital (PB) was given as a tumor promoter in drinking water (0.05%) from 4 weeks after DEN administration until sacrifice. Arrows indicate HCCs.

(E) HCC multiplicity, size, and incidence in WT male mice treated as in (D).

Results are means ± SD (n = 10–13). *p < 0.05, **p < 0.01, ***p < 0.001 denote significant differences between the groups. See also Figure S1 and S2.

to decrease cell death and enhance cell proliferation. These findings suggest that alterations in signal transduction pathways that modulate hepatoma cell proliferation independently of liver damage and compensatory proliferation may underlie the tumor-promoting effect of obesity especially at later time points.

To further examine this notion and separate effects of obesity on tumor growth and progression from effects on tumor initiation, we transplanted established hepatoma cells (derived from a DEN-induced HCC) into 2-month-old lean mice that were kept after tumor cell inoculation on either LFD or HFD for 4 weeks. We also inoculated the same number of hepatoma cells into 8-month-old mice that were kept on HFD for the preceding 6 months or were genetically obese (Lep^{Ob}). In all cases, tumor growth was monitored over the course of 4 weeks after inoculation. The greater the degree of host obesity, the faster the tumors grew, reaching the largest size in Lep^{Ob} mice (Figure 3). We also treated some of the mice with the JAK inhibitor AG490 (Eriksen et al., 2001) to inhibit STAT3 activation (see below). AG490 exerted a stronger inhibitory effect on tumor growth in 2-month-old mice kept on HFD than in mice kept on LFD (Figures 3B and 3C) and as expected inhibited STAT3 phosphorylation (Figure 3D).

Obese HCC-Bearing Mice Exhibit Elevated STAT3 and ERK Activation and Liver Inflammation

To identify signaling pathways responsible for enhanced hepatoma cell survival and proliferation, we first examined the effect

unless treated with PB after DEN administration. Furthermore, the tumor-promoting effect of dietary obesity was at least as strong as that of PB, a bona fide liver tumor promoter (Ward et al., 1986).
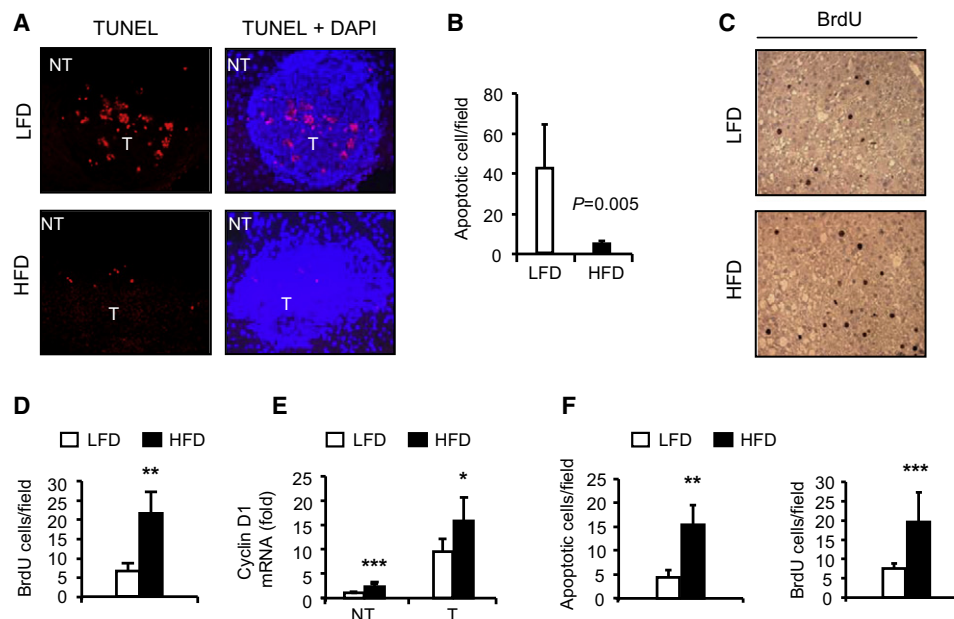
Obesity Is Associated with Increased Cancer Cell Proliferation

Enhanced DEN-induced hepatocyte death, as observed in IKKβ and p38α liver-specific knockout mice ($Ikk\beta^{\Delta hep}$, $p38\alpha^{\Delta hep}$), or spontaneous liver damage in $Nemo/Ikk\gamma^{\Delta hep}$ mice are associated with enhanced compensatory proliferation and augmented HCC development (Hui et al., 2007; Luedde et al., 2007; Maeda et al., 2005; Sakurai et al., 2008). As mentioned above, DEN administration to obese mice induced more liver damage than in lean mice. Nonetheless, HCCs in obese mice exhibited reduced apoptotic cell death relative to HCCs in lean mice (Figures 2A and 2B). Concurrently, HCCs in obese mice exhibited more proliferating cells than HCCs in lean mice (Figures 2C and 2D), as well as elevated cyclin D1 mRNA expression (Figure 2E). Thus, despite the early increase in both DEN-induced apoptosis and compensatory proliferation in obese mice given DEN at 16 weeks of age (Figure 2F), the long-term effect of obesity on HCC cell kinetics is

Figure 2. Obesity in HCC-Bearing Mice Is Associated with Increased Cancer Cell Proliferation and Reduced Apoptosis

HCC-bearing (protocol #1) male mice were subjected to histological and biochemical analyses at 9 months of age.

(A) Obesity reduces cell death in HCCs. Paraffin-embedded liver sections were TUNEL labeled (left panels) and counterstained with DAPI (right panels). Note the presence of apoptotic cells in the tumor (T) area in lean mice. Magnification: 20×.

(B) Numbers of apoptotic cells per field in (A) was determined by Image Tool software (n = 5).

(C) Obesity-enhanced cell proliferation in HCCs. Mice were BrdU pulsed 2 hr prior to sacrifice. Paraffin-embedded liver sections were stained with anti-BrdU antibody.

(D) Numbers of BrdU-positive proliferating cells in (C) was determined with Image Tool software (n = 5).

(E) Expression of cyclin D1 mRNA in nontumor liver (NT) and tumors (T) was determined by qRT-PCR (n = 5–6). Values were normalized to cyclophilin mRNA.

(F) Mice kept on LFD or HFD for 10 weeks were injected with DEN (100 mg/kg) and their livers were isolated 48 hr after DEN administration and 2 hr after a BrdU pulse. Paraffin-embedded liver sections were subjected to TUNEL and BrdU staining and positive cells were counted as above (n = 5).

All values represent means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 denote significant differences between the groups.

of obesity on several protein kinases known to be affected by metabolic state. As expected (Tremblay et al., 2007; Um et al., 2004), obesity was associated with decreased AKT phosphorylation and increased phosphorylation of the mTOR target S6 kinase and its substrate ribosomal protein S6 (Figure 4A). This was observed in both normal livers and HCCs, strongly suggesting that counter to a previous hypothesis (Calle and Kaaks, 2004), AKT activation, driven by insulin or IGF-1, is unlikely to be responsible for enhanced cell survival and proliferation in HCCs of obese individuals.

We next examined the effect of obesity on several signaling molecules affected by inflammation and known to modulate HCC development (Hui et al., 2007; Maeda et al., 2005; Sakurai et al., 2006, 2008). Consistent with previous findings (Hirosumi et al., 2002; Solinas et al., 2007), obese mice exhibited elevated JNK activity in liver and even a larger increase in JNK phosphorylation in HCCs (Figures 4B and 4C). HCCs in obese mice also exhibited greatly elevated ERK phosphorylation relative to HCCs of lean mice, but p38 phosphorylation was not altered in obese liver and was even reduced in HCCs of obese mice (Figures 4B and 4C). Remarkably, both nontumor liver tissue and HCCs from obese mice displayed a substantial increase in STAT3 phosphorylation, indicative of activation of this oncogenic transcription factor (Figures 4B and 4C).

Consistent with the increase in STAT3 activation, we found that obese mice exhibited elevated circulating IL-6 (Figure 4D), a potent STAT3-activating cytokine (Kamimura et al., 2003). Elevated IL-6 mRNA was observed in both nontumor liver and HCCs of obese mice (Figure 4E). Obesity also enhanced expression of TNF and IL-1β mRNAs (Figures 4F and 4G). The amount of TNF protein was also greatly elevated in normal liver and HCC from obese mice (Figure 4H). Elevated IL-6, TNF, and IL-1β expression was also observed in obese female tumor-bearing mice (Figures S3A–S3D). Consistent with elevated inflammatory cytokines, livers from obese mice contained higher amounts of macrophages and other leukocytes (Figures S3E–S3I).

Enhanced IL-6 Production Is Required for Obesity-Induced Tumor Promotion

Given the marked increase in STAT3 phosphorylation and circulating IL-6 in obese mice and their tumors, and knowing that IL-6 functions as a downstream mediator for both IL-1 and TNF (Kamimura et al., 2003), we examined whether IL-6 is an important component of the tumor-promoting mechanism activated by obesity. To this end, we subjected WT and *IL6*^{−/−} mice to protocol #1 (Figure S1A), in which DEN is given to 2-week-old mice followed by either LFD or HFD. Remarkably, *IL6*^{−/−} male mice, which developed much fewer HCCs than WT mice when
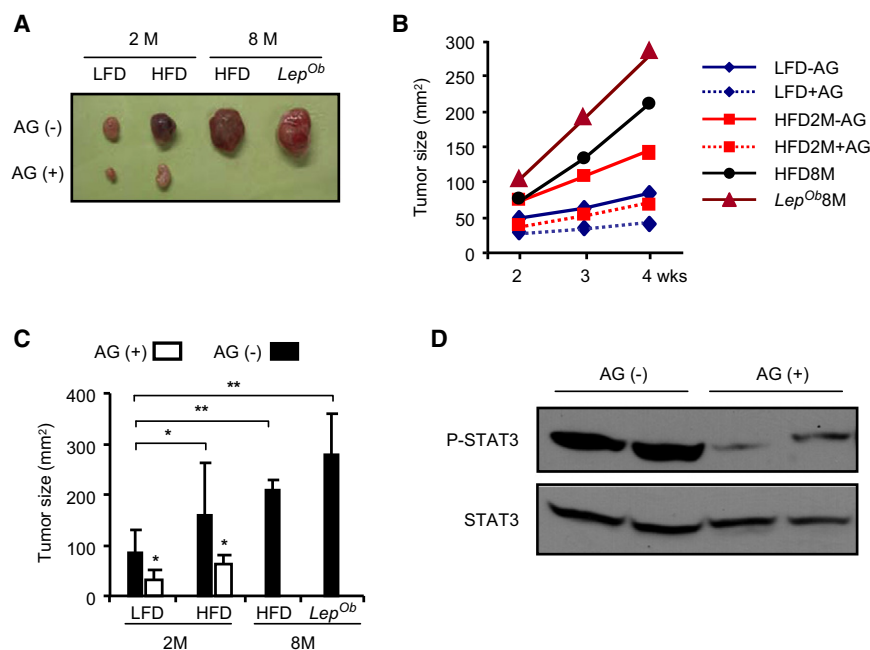
Figure 3. Obesity Promotes Tumorigenic Growth of Hepatoma Cells

(A) Two-month-old lean mice (2 M) were subcutaneously injected with established hepatoma cells (2.5×10^6 cells/mouse) and kept on LFD or HFD for 4 weeks after inoculation with (AG+) or without (AG−) AG490 (0.5 mg/mouse/day), a JAK inhibitor that blocks STAT3 activation. The same number of hepatoma cells was also transplanted into 8-month-old mice that were kept on HFD for the preceding 6 months or were genetically obese (Lep^{Ob}). Tumor growth was monitored by measurement with a caliper once a week, from week 2 to week 4. The images show representative solid tumors dissected at week 4.

(B) Tumor growth measured over the course of a 2 week period in mice described in (A). The results are means for 3–6 mice per group.

(C) Tumor sizes at week 4 post-inoculation are shown as a histogram. Results are means ± SD (n = 3–6).

(D) Tumors grown in mice given HFD without (AG−) or with (AG+) AG490, administered as above, were collected at week 4 post-inoculation and homogenized, and STAT3 phosphorylation and expression were analyzed by immunoblotting. All values represent means ± SD. *$p < 0.05$, **$p < 0.01$ denote significant differences between the groups.

kept on LFD, an observation consistent with previous findings (Naugler et al., 2007), hardly exhibited any augmentation of tumor multiplicity, size, and incidence when placed on HFD (Figures 5A and 5B). Importantly, the HCC load in $IL6^{-/-}$ male mice is identical to that of WT females (Naugler et al., 2007), but unlike WT females, which produce more IL-6 when rendered obese (Figures S3A and S3B) and develop more HCC, no significant increase in tumor load was seen in obese $IL6^{-/-}$ males (Figure 5C). Although $IL6^{-/-}$ males did not gain weight as rapidly as WT males, they reached almost the same weight as WT mice after 8 months on HFD (Figure 5D) and exhibited elevated serum TG (Figure 5E). $IL6^{-/-}$ males also showed increased liver TG content after placement on HFD, although the extent of liver TG accumulation was lower than in WT males. $IL6^{-/-}$ male mice also presented with elevated serum insulin after placement on HFD, but the increase was not as robust as in obese WT males (Figure 5E).

These data suggest that obesity-associated chronic elevation in IL-6, a tumor-promoting cytokine whose expression can be induced by both TNF and IL-1, is an important contributing factor to liver tumorigenesis.

To better understand how the absence of IL-6 prevents obesity-induced tumor promotion, we examined its effect on stress/inflammation- and metabolism-responsive signaling molecules. The absence of IL-6 prevented the obesity-induced increase in JNK and ERK phosphorylation in nontumor liver and HCCs and completely reversed the decrease in p38 phosphorylation previously seen in HCCs of obese mice (Figure 5F and Figure S4A; compare to Figures 4B and 4C). As expected, the absence of IL-6 prevented STAT3 phosphorylation in liver and HCCs of obese mice (Figure 5F and Figure S4A). Interest-

ingly, the IL-6 deficiency also prevented much of the obesity-induced increase in S6 phosphorylation in both liver and HCCs and partially attenuated the decrease in AKT phosphorylation (Figure 5F and Figure S4A). These finding suggest that IL-6 is not only required for STAT3, ERK, and JNK activation in the obese liver, but it also contributes (indirectly, see below) to the alterations in AKT and mTOR-S6K signaling.

TNF Receptor Signaling Is Required for Obesity-Induced Tumor Promotion

In addition to IL-6, HFD increases expression of TNF, a proinflammatory cytokine that stimulates IL-6 production. We previously found that TNF signaling via its type 1 receptor, TNFR1, is not required for DEN-induced hepatocarcinogenesis in lean mice (Naugler et al., 2007). Nonetheless, given the dramatic increase in liver TNF production in obese mice (Figure 4H), we examined whether TNFR1 signaling contributes to obesity-induced tumor promotion. Remarkably, ablation of TNFR1 almost completely abolished obesity-enhanced HCC development without affecting HCC induction in lean mice (Figures 6A and 6B). $TNFR1^{-/-}$ mice, however, gained almost as much weight as WT mice (Figure 6C). The rate of weight gain was also very similar in the two strains.

Ablation of TNFR1 reduced JNK and p38 phosphorylation in both lean and obese mouse livers and prevented the increase in JNK activity associated with HCC development (Figure 6D and Figure S4B). In addition, $TNFR1^{-/-}$ mice did not show the obesity-induced increase in STAT3 and ERK phosphorylation. TNFR1 ablation also prevented the decrease in AKT phosphorylation seen in HFD-fed WT mice and attenuated the changes in S6 phosphorylation (Figure 6D and Figure S4B).
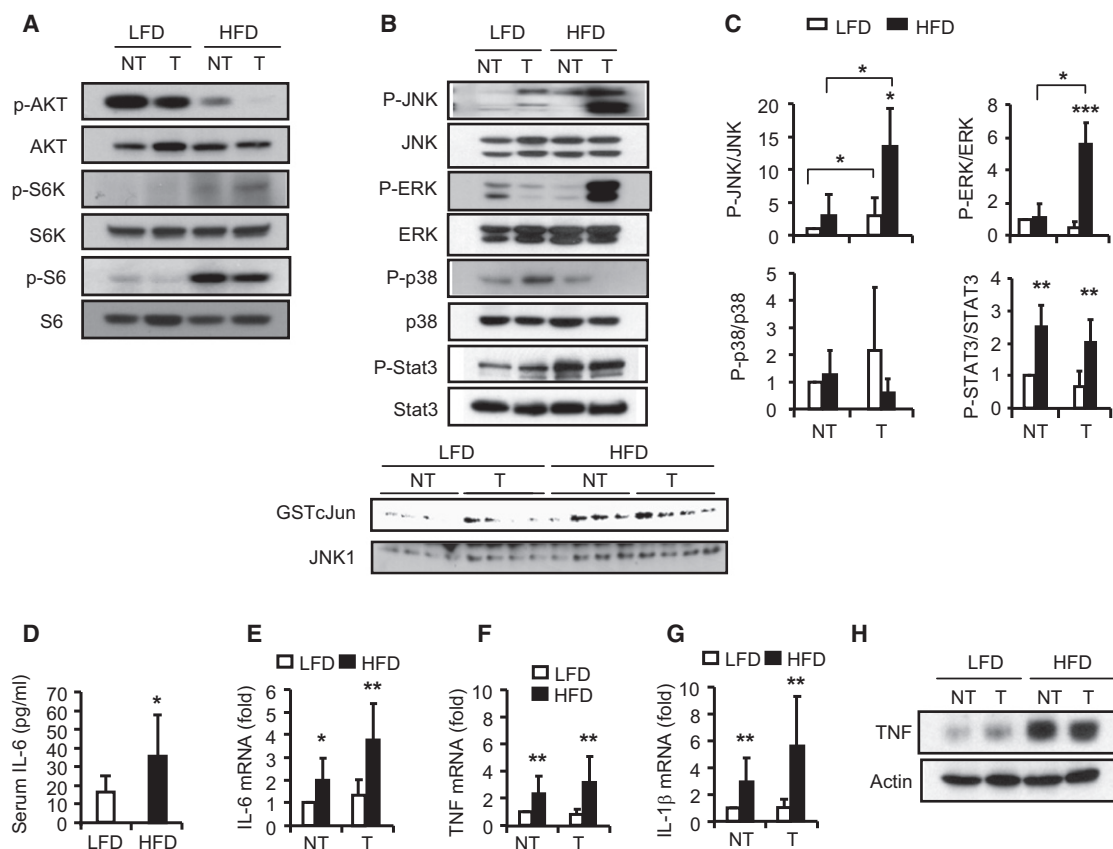
Figure 4. Obese HCC-Bearing Mice Exhibit Activated STAT3 and Elevated Expression of Inflammatory Cytokines

The HCC-bearing mice from Figure 2 were subjected to additional biochemical and gene expression analyses.

(A) Activation state of metabolically regulated protein kinases. Nontumor (NT) liver and HCC (T) were lysed and gel separated, and the activation (phosphorylation) states of AKT and S6 kinase (S6K) were examined by immunoblotting.

(B) Nontumor (NT) liver and HCC (T) were analyzed for phosphorylation of JNK, ERK, p38, and STAT3 by immunoblotting as above. The bottom panel shows JNK kinase assays in nontumor liver and HCCs of four different mice per dietary group.

(C) The results of several immunoblots similar to the one shown in (B), each representing a different mouse, were quantitated using Image J software and mean values were determined (n = 3–4).

(D) Serum IL-6 in HCC-bearing mice was determined by ELISA (n = 10–12).

(E–G) Relative amounts of IL-6 (E), TNF (F), and IL-1β (G) mRNAs in nontumor (NT) liver and HCC (T) were determined by qRT-PCR and normalized to cyclophilin mRNA (n = 6–8).

(H) TNF amounts in nontumor liver (NT) and HCC (T) were examined by immunoblotting of tissue lysates.

All values represent means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 denote significant differences between the groups. See also Figure S3.

TNFR1 Signaling and IL-6 Promote Steatohepatitis

We further examined $IL6^{-/-}$ and $TNFR1^{-/-}$ tumor-bearing mice to identify a common mechanism that could explain their resistance to obesity-induced tumor promotion. As expected, the absence of TNFR1 reduced the obesity-induced increase in IL-6 production, resulting in lower-circulating IL-6 and less IL-6 mRNA in liver and in HCCs of obese mice (Figures 7A and 7B). Conversely, the absence of IL-6 reduced the obesity-induced increase in TNF production in tumor-bearing mice (Figure 7C). The absence of either IL-6 or TNFR1 in tumor-bearing mice reduced HFD-induced liver lipid accumulation (Figures 7D and 7E). The absence of either IL-6 or TNFR1 also reduced macrophage and neutrophil accumulation in livers of HFD-fed mice (Figures 7F and 7G). We therefore conclude that both IL-6 and TNF signaling via TNFR1 are important for liver lipid accumulation (hepatosteatosis) and fat-induced liver inflammation (steatohepatitis), which together define NAFLD, a condition that greatly increases the risk of HCC development (El-Serag and Rudolph, 2007; Parekh and Anania, 2007).

DISCUSSION

Overweight and obesity greatly increase HCC risk, especially in men (Calle and Kaaks, 2004; Calle et al., 2003). Despite the magnitude of the effect and the very large number (900,000) of individuals on which the epidemiological study that provided this insight was based, the mechanisms by which obesity increases risk of death from HCC and other cancers remained unknown. We now describe that genetic or dietary obesity greatly enhances the development of chemically induced HCC
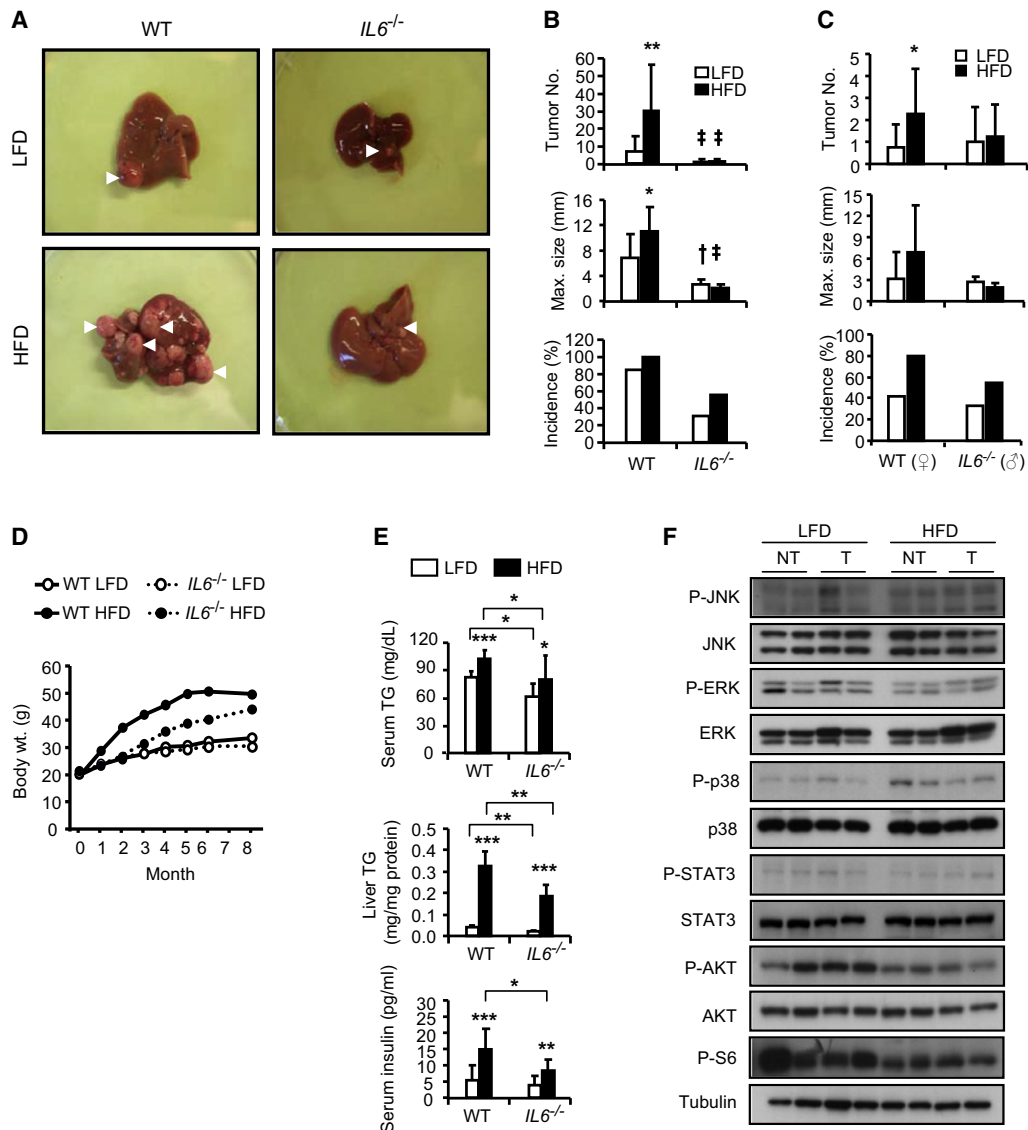
Figure 5. Enhanced IL-6 Production Is Required for Obesity-Induced Tumor Promotion

(A) Livers of WT and IL-6-deficient ($IL6^{-/-}$) male mice that were kept on LFD or HFD after the administration of DEN (25 mg/kg) at 14 days of age.

(B) HCC multiplicity, size, and incidence (n = 10–12) in DEN-administered male mice of the indicated genotype kept on either LFD (□) or HFD (■).

(C) HCC multiplicity, size, and incidence (n = 10–12) in DEN-administered WT females or $IL-6^{-/-}$ males of the indicated genotype kept on either LFD (□) or HFD (■).

(D) Body weight gain during tumor development in WT and $IL-6^{-/-}$ male mice kept on LFD or HFD after DEN administration.

(E) Serum and liver triglycerides (TG) in mice from (D) were determined by a colorimetric assay at 9 months of age. Serum insulin was determined by ELISA at the same age (serum, n = 8–12; liver, n = 5).

(F) HCCs (T) and nontumor (NT) liver tissue from $IL6^{-/-}$ male mice kept on either LFD or HFD were isolated, homogenized, and subject to immunoblot analysis to determine the phosphorylation state of the indicated proteins. Shown are the results from two separate tumors for each condition.

All values are means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 denote significant differences between the groups. †p < 0.05, ‡p < 0.01 presents significant differences from WT mice. See also Figure S4.

in laboratory mice. By administering a chemical carcinogen (DEN) to adult mice in which it fails to induce HCC on its own, we demonstrated that obesity is a bona fide tumor promoter whose effect is at least as strong as that of the validated liver tumor promoter phenobarbital. Furthermore, we found that the tumor-promoting effect of obesity in HCC depends to a large

extent on the low-grade inflammatory response it induces, which involves elevated production of TNF and IL-6, both of which are tumor-promoting cytokines (Lin and Karin, 2007).

While our work was being completed, it was described that consumption of HFD resulted in increased formation of preneoplastic lesions in response to DEN administration in rats

A

B

C

D
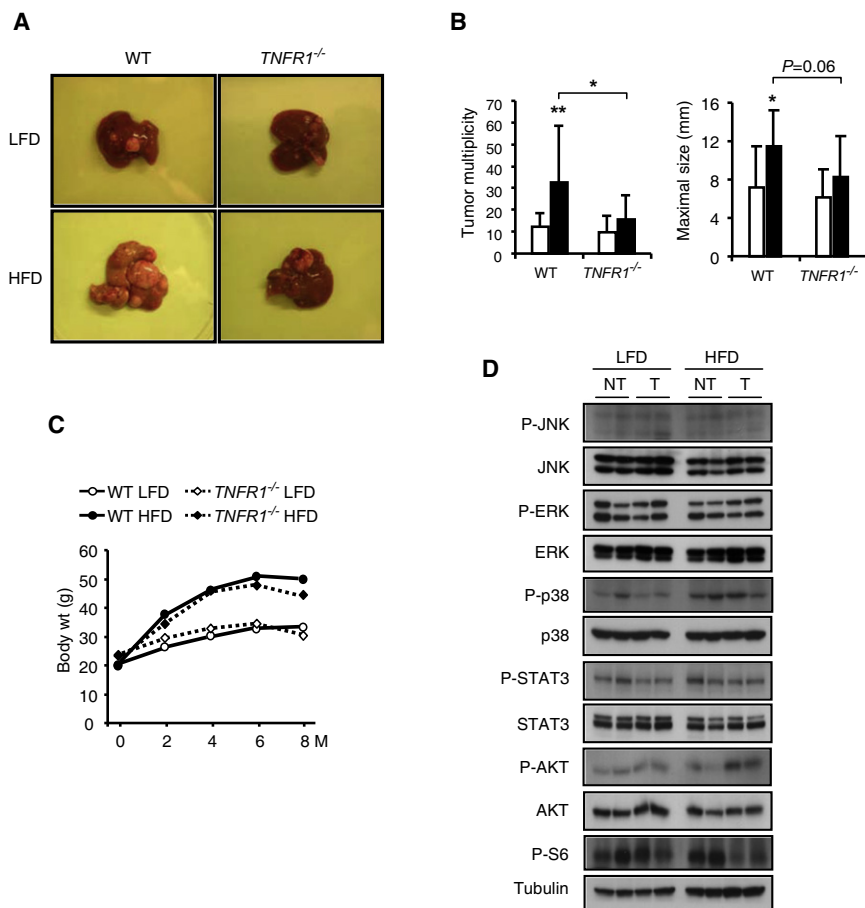
Figure 6. TNFR1 Signaling Is Required for Obesity-Induced Tumor Promotion

(A) Livers of WT or TNFR1-deficient male mice kept on LFD or HFD after DEN administration (25 mg/kg) at 14 days of age.

(B) Tumor multiplicity and maximal sizes in DEN-administered male mice of the indicated genotypes kept on either LFD (□) or HFD (■) (n = 10–13).

(C) Body weight gain during tumor development in WT or $TNFR1^{-/-}$ male mice kept on LFD or HFD after DEN administration.

(D) Nontumor (NT) liver and HCC (T) from the mice described in (A) were isolated, lysed, and gel separated, and the phosphorylation states of the indicated proteins were examined by immunoblotting. Shown are the results for two separate mice per condition.

by consumption of HFD for 10 weeks and then challenged with DEN. This elevated hepatotoxic response may contribute to the tumor-promoting effect of obesity. We also found that obese mice accumulate more ROS both in nontumor liver tissue and in HCCs (Figure S2G). However, this persistent ROS accumulation, which is also seen during chronic HCV infection (Wang and Weinman, 2006), does not lead to increased cell death in HCCs of obese mice. By contrast, HCCs in obese mice display substantially lower apoptotic rates than HCCs in lean mice (Figure 2A). Despite the reduction in apoptosis, HCCs in obese mice contain more proliferating cells (Figures 2C and 2D), suggesting that enhanced compensatory proliferation driven by hepatocyte death does not account for mid to late tumor promotion in obese mice. Instead, mid to late tumor promotion in obese mice correlates with enhanced proliferation and reduced death of malignant hepatoma cells. Indeed, fully initiated hepatoma cells exhibit higher tumor growth rates when transplanted into obese mice, and this accelerated tumor growth is slowed down by administration of a JAK inhibitor that prevents STAT3 activation. Notably, IL-6 signaling through ERK and STAT3 can reduce epithelial cell apoptosis while stimulating cell proliferation, as recently found in colitis-associated cancer (Grivennikov et al., 2009).

A survey of different signaling molecules in human HCC revealed frequent activation of ERK and STAT3, especially in HCCs with poor prognosis, as well as reduced p38 activation, relative to the surrounding liver tissue (Calvisi et al., 2006). Similar changes were observed in HCCs of WT obese mice (Figures 4A–4C). We have also confirmed frequent STAT3 activation in human HCC and, using liver-specific STAT3 knockout mice ($Stat3^{\Delta hep}$), found that this major transcriptional target for IL-6 is required for HCC development (He et al., 2010). Furthermore, expression of IL-6, a major STAT3 activator in the liver, is elevated in cirrhosis and HCC (Tilg et al., 1992; Trikha et al., 2003). Elevated serum

(Wang et al., 2009). However, these rats were not evaluated for HCC and the mechanism underlying increased formation of pre-neoplastic lesions was not determined. Another recent study using $Nemo/Ikk\gamma^{\Delta hep}$ mice, which spontaneously develop liver damage, hepatosteatosis, and HCC on a normal diet (Luedde et al., 2007), found that consumption of HFD enhanced the development of hepatosteatosis and ensuing liver damage and accelerated the appearance of liver tumors (Wunderlich et al., 2008). However, the pre-existing hepatosteatosis and spontaneous liver damage in $Nemo/Ikk\gamma^{\Delta hep}$ mice that are kept on LFD prevented the conclusive demonstration that obesity per se rather than chronic liver damage caused by the absence of NF-κB is responsible for tumor promotion. Furthermore, the mechanism by which obesity accelerates HCC development in these mice was not determined. Enhanced liver tumorigenesis is not unique to $Nemo/Ikk\gamma^{\Delta hep}$ mice and was also seen upon DEN administration to $Ikk\beta^{\Delta hep}$ and $p38\alpha^{\Delta hep}$ mice, which do not manifest spontaneous liver damage or hepatosteatosis (Hui et al., 2007; Maeda et al., 2005; Sakurai et al., 2008). It was proposed that enhanced liver damage driven by ROS accumulation, which gives rise to compensatory proliferation of initiated hepatocytes, may explain augmented HCC development in all of these mouse strains, which are more susceptible to DEN-induced hepatotoxicity (Sakurai et al., 2006, 2008). Enhanced liver damage is also seen in mice that have been rendered obese

engagement by TNF contributes to obesity-induced IL-6 production (Figures 7A and 7B), prevents obesity-promoted hepatocarcinogenesis without affecting DEN-induced hepatocarcinogenesis in lean mice.

Although TNF and IL-6 may not be the only mediators responsible for obesity-induced tumor promotion, it is well-established that their production is elevated in obese mice due to the low-grade inflammatory response caused by lipid accumulation (Arkan et al., 2005; Hotamisligil, 2006; Shoelson et al., 2007; Solinas et al., 2007). In humans, IL-6 is thought to be produced by adipose tissue during noninflammatory conditions and is therefore considered to be an adipokine (Fried et al., 1998; Mohamed-Ali et al., 1997). Not surprisingly, IL-6 in serum shows positive correlation with BMI (Bastard et al., 2000). IL-6 is also produced by Kupffer cells where it is induced in response to hepatocyte death, which results in IL-1 release (Sakurai et al., 2008). Not only IL-1, but TNF, whose production is elevated in the obese liver, also leads to IL-6 production, and our results reveal a substantial reduction in obesity-induced IL-6 in $TNFR1^{-/-}$ mice. Regardless of its source, it is established that IL-6 can propagate inflammation (Kamimura et al., 2003) and

IL-6 also correlates with a higher risk of progression from chronic viral hepatitis to HCC (Nakagawa et al., 2009; Wong et al., 2009). A role for IL-6 in HCC development was first suggested by our findings that differential IL-6 production following DEN-induced liver damage accounts for the gender bias in HCC induction; female mice produce 2-fold less IL-6 after DEN challenge than males and therefore develop less HCC (Naugler et al., 2007). Here we show that $IL6^{-/-}$ male mice no longer respond to the tumor-promoting effect of obesity, but female mice that can produce more IL-6 when placed on HFD are susceptible to obesity-induced tumor promotion, even though their basal HCC induction rate is very low and similar to that of IL-6-deficient males. Furthermore, inactivation of TNFR1, a receptor whose

stimulate triglyceride secretion (Nonogaki et al., 1995). However, the complete absence of IL-6 in mice was reported to have controversial effects on adiposity. Whereas one study found that $IL6^{-/-}$ mice became spontaneously obese as they got older (Wallenius et al., 2002), another study failed to find any basal alteration in body weight and fat deposition in $IL6^{-/-}$ mice kept on LFD (Di Gregorio et al., 2004). When placed on HFD, $IL6^{-/-}$ mice were found to gain weight slower than WT mice but eventually did become obese (Di Gregorio et al., 2004) as seen in our study (Figure 5D). Thus the absence of obesity-induced tumor promotion in $IL6^{-/-}$ mice is not due to their failure to gain weight. Importantly, however, although total body weight and serum triglycerides were still elevated, liver lipid accumulation was substantially reduced in both $IL6^{-/-}$ and $TNFR1^{-/-}$ mice (Figures 7D and 7E). Thus, both TNF and IL-6 contribute to the maintenance of hepatosteatosis. Furthermore, the absence of TNFR1 or IL-6 prevented steatohepatitis, resulting in much lower amounts of HFD-induced macrophage and neutrophil accumulation in the liver relative to WT mice (Figures 7F and 7G). In humans, steatohepatitis is the more severe manifestation of NAFLD and it, rather than insulin resistance, is likely to be the major contributor to obesity-induced tumor promotion in the liver.

In addition to propagation of steatohepatitis, IL-6 leads to STAT3 activation in hepatocytes (Naugler et al., 2007), and STAT3 can stimulate the proliferation and progression of initiated hepatocytes (He et al., 2010) and thus could be a molecular mediator of tumor promotion. Given the involvement of STAT3 in several other cancers (Bromberg and Wang, 2009) and the elevation in circulating IL-6 in obese and overweight individuals (Bastard et al., 2000), it is likely that IL-6 and STAT3 may contribute to the general enhancement of cancer risk by high BMI. However, obesity, elevated IL-6, and activated STAT3 are unlikely to cause cancer on their own. Even genetic alterations that result in constitutive STAT3 activation in hepatocytes only cause benign hepatic adenomas, unless combined with oncogenic mutations (Rebouissou et al., 2009). Most likely, chronic activation of the IL-6-STAT3 axis increases the likelihood of expansion and further progression of transformed hepatocytes that have acquired oncogenic mutations upon exposure to environmental and dietary carcinogens. Notably, most xenobiotics are metabolized within the liver, but sometimes this metabolism results in the activation, rather than elimination, of procarcinogens (Sheweita and Tilmisany, 2003).

IL-6-induced STAT3 activation, however, is unlikely to be the only tumor-promoting mechanism stimulated by obesity. It was suggested that another tumor-promoting mechanism triggered by obesity is enhanced AKT activation caused by the high concentrations of circulating insulin and IGF-1 in overweight and obese individuals (Calle and Kaaks, 2004). However, obesity results in insulin resistance and actually leads to inhibition of AKT activation as shown above. Thus AKT activation is unlikely to contribute to enhanced HCC development in obese mice. A more relevant player whose contribution merits further and dedicated studies is mTOR, whose activity is elevated in the obese liver (Figure 4A) and which is known to be an important regulator of cell and tumor growth (Guertin and Sabatini, 2007). The mouse models described above should be suitable for such studies.

EXPERIMENTAL PROCEDURES

Mice, Diet, Tumor Induction, and Analysis

IL-6-deficient ($IL6^{-/-}$), TNFR1-deficient ($TNFR1^{-/-}$), and leptin-deficient (Lep^{Ob}) mice on a C57BL/6 background were used along with corresponding age-matched WT mice. All mice were maintained in filter-topped cages on autoclaved regular chow diet (low-fat diet; LFD, composed of 12% fat, 23% protein, 65% carbohydrates based on caloric content) or high-fat diet (HFD, composed of 59% fat, 15% protein, 26% carbohydrates based on caloric content; BioServ) and water at UCSD according to NIH Guidelines.

In the first DEN-induced HCC model (Figure S1A, protocol #1), DEN (25 mg/kg) was injected intraperitoneally (i.p.) into 14-day-old mice. After 4 weeks, mice were separated into two dietary groups and fed either LFD or HFD until sacrificed. In the second HCC model (Figure S1B, protocol #2), mice were fed either LFD or HFD for 10 weeks prior to DEN administration (i.p., 80 mg/kg) at 16 weeks of age, until sacrificed. Four weeks after DEN administration, mice received water with or without PB (0.05%, Sigma) until sacrificed. Serum insulin, IL-6, and TNF were measured by ELISA (eBioscience) and ALT was detected using Biotron kit. Tumors in each liver lobe were counted and measured with a caliper. Tumor and nontumor tissue was collected and rapidly frozen for biochemical, histological, and immunochemical analysis. In the HCC induction studies, mice were not treated with any hormone or drug except when indicated (PB administration). In the transplant study (Figure 3), mice did not receive any drug or hormone other than AG490 (LC Labs) when indicated.

Histological, Immunohistochemical, and Immunofluorescent Assay

Liver tissue was fixed in 10% formalin or embedded in Tissue-Tek OCT compound (Sakura Finetek) for paraffin and frozen block preparation, respectively. Paraffin-embedded liver tissue was used for H&E, TUNEL (Roche), and immunohistochemical staining as described (Sakurai et al., 2008). TUNEL- and BrdU-positive cells were quantified using Image tool. Frozen tissue sections were stained with oil-red O (ORO) for lipid detection and dihydroethidine (DHE) to detect ROS accumulation (Maeda et al., 2005).

Triglyceride, Insulin, and Glucose Measurement

Mice were fasted for 6 hr, after which their blood was analyzed for insulin (ELISA: Crystal Chem Inc) and TG content (calorimetric assay: Diagnostic Chemicals Limited). Liver TG was extracted in chloroform/methanol (2:1, v/v) (Folch et al., 1957) and measured as above. For glucose tolerance test, mice were injected with glucose (2 g/kg, i.p.) after 6 hr of fasting. Blood glucose was measured using glucose meter (LifeScan).

Real-Time Quantitative PCR, Immunoblotting, and Kinase Assays

Total liver and HCC RNA was extracted with RNase Plus kit (QIAGEN) and cDNA was synthesized using SuperScript II kit (Invitrogen). Real-time PCR was performed using SYBR green (Bio-Rad) on a Bio-Rad iQ5 machine. Values were normalized to cyclophilin mRNA amount. Liver lysates prepared in standard RIPA buffer were gel separated and proteins were transferred to PVDF membranes (Whatman). Membranes were probed with primary antibodies and secondary HRP-conjugated antibodies (GE Healthcare UK) and developed using ECL detection reagent (Pierce). Protein band intensity was quantified using Image J software. JNK immunocomplex kinase assay was performed using GST-cJun(1-79) as a substrate and a JNK1/2 antibody (Pharmingen) as described (Solinas et al., 2007).

Statistical Analysis

Data are presented as means ± standard deviation. Statistical significance was calculated with a Student's t test. Significance was accepted at the level of $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.cell.2009.12.052.

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