The role of JNK in the development of hepatocellular carcinoma

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Keywords
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Comments
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The role of JNK in the development of hepatocellular carcinoma

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The cJun NH2-terminal kinase (JNK) signal transduction pathway has been implicated in the growth of carcinogen-induced hepatocellular carcinoma. However, the mechanism that accounts for JNK-regulated tumor growth is unclear. Here we demonstrate that compound deficiency of the two ubiquitously expressed JNK isoforms (JNK1 and JNK2) in hepatocytes does not prevent hepatocellular carcinoma development. Indeed, JNK deficiency in hepatocytes increased the tumor burden. In contrast, compound JNK deficiency in hepatocytes and nonparenchymal cells reduced both hepatic inflammation and tumorigenesis. These data indicate that JNK plays a dual role in the development of hepatocellular carcinoma. JNK promotes an inflammatory hepatic environment that supports tumor development, but also functions in hepatocytes to reduce tumor development.

[Keywords: JNK; partial hepatectomy; hepatocellular carcinoma]

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The cJun NH2-terminal kinase (JNK) pathway is implicated in tumor development [Davis 2000]. Targets of JNK signaling include members of the activating protein 1 (AP1) transcription factor group [e.g., cJun, JunB, JunD, and related proteins]. These transcription factors function within a regulatory network that controls multiple aspects of cellular physiology, including proliferation [Eferl and Wagner 2003]. Studies of murine embryonic fibroblasts [MEFs] demonstrate that loss of JNK causes major defects in cellular proliferation [Tournier et al. 2000] and AP1-dependent gene expression [Ventura et al. 2003]. These effects of JNK are mediated, in part, by the AP1 transcription factor. Indeed, cJun phosphorylation is required for normal serum-stimulated cell growth [Behrens et al. 1999]. The JNK signaling pathway therefore represents an important regulatory mechanism that can control growth. Moreover, dysregulated JNK may contribute to tumor development [Davis 2000].

Studies of the role of the JNK and cJun in the liver have confirmed the importance of this signaling pathway in growth regulation. Thus, cJun-deficient mice [Behrens et al. 2002; Stepniak et al. 2006], JNK1-deficient mice [Hui et al. 2008], and mice treated with a JNK inhibitor [Schwabe et al. 2003] exhibit major defects in liver regeneration following partial hepatectomy [PHx]. Furthermore, both cJun-deficient mice [Eferl et al. 2003] and JNK1-deficient mice [Sakurai et al. 2006; Hui et al. 2008] are protected against the development of hepatocellular carcinoma [HCC] following exposure to the carcinogen diethylnitrosamine [DEN]. The critical role of JNK in HCC has been confirmed by pharmacological inhibition of JNK and studies of human HCC cells [Hui et al. 2008].

The mechanism of JNK and cJun signaling in the liver that contributes to regeneration and HCC is unclear, but down-regulation of the proliferation inhibitor p21CIP1 and up-regulation of the growth promoter cMyc appear to be critical factors [Stepniak et al. 2006; Hui et al. 2008]. An alternative mechanism for the contribution of JNK to HCC is represented by the role of compensatory proliferation in hepatic tumor development [Fausto 1999]. Reduced hepatocyte death in JNK1-deficient mice results in reduced compensatory proliferation and suppression of HCC [Sakurai et al. 2006]. These mechanisms are not mutually exclusive, and it is possible that JNK plays multiple roles in HCC, including regulation of cell death and gene expression. Nevertheless, a common theme among these mechanisms is that JNK plays a critical role in hepatocytes that is required for HCC development.

The purpose of this study was to examine the role of JNK in the development of HCC. Specifically, we tested whether JNK in hepatocytes contributes to tumor formation. Previous studies have focused on an analysis of Jnk1−/− mice [Sakurai et al. 2006; Hui et al. 2008]. Here we report the analysis of mice with tissue-specific deficiency of JNK and mice with compound deficiency of both JNK1 and JNK2. Our analysis demonstrates that...
compound JNK deficiency in hepatocytes increases the development of HCC. JNK therefore functions in liver parenchymal cells to reduce tumor development. We show that the protumorigenic effects of JNK on HCC are associated with inflammation and require JNK function in nonparenchymal cells.

Results

Compound JNK deficiency in hepatocytes does not prevent liver regeneration following PHx

PHx causes JNK activation and a robust regeneration response that results in rapid restoration of liver mass (Westwick et al. 1995). It has been reported that Jnk1−/− mice exhibit a defect in the regeneration response to PHx (Hui et al. 2008). In initial studies, we compared hepatic regeneration in control mice, Jnk1−/− mice, and Jnk2−/− mice following PHx. This analysis demonstrated similar hepatic regeneration in control and Jnk2−/− mice, but hepatic regeneration was suppressed in Jnk1−/− mice (Supplemental Fig. S1). These data indicate that JNK1 may play an important role in hepatocyte proliferation (Hui et al. 2008).

The Jnk1 and Jnk2 genes are both expressed in hepatocytes (Davis 2000). Consequently, the reduced hepatic regeneration detected in Jnk1−/− mice (Supplemental Fig. S1) is not caused by loss of total JNK in hepatocytes. These considerations indicated that studies of hepatic regeneration in mice with compound ablation of Jnk1 plus Jnk2 are required. We employed a conditional gene ablation strategy using Alb-Cre transgenic mice to create animals with compound deficiency of JNK1 plus JNK2 in hepatocytes (Das et al. 2009). Control HWT mice (Alb-Cre+/−) and JNK-deficient HΔJNK mice (Alb-Cre+/− Jnk1LoxP/LoxP Jnk2−/−) were examined following PHx or a sham surgical procedure (Fig. 1A).

Biochemical analysis of the liver of HWT and HΔJNK mice at 48 h post-PHX demonstrated that JNK deficiency did not significantly change the expression of cjun, JunB, or Cyclin D1 mRNA, but a modest reduction in JunD
mRNA expression was detected in the liver of H\textsuperscript{JNK\textsuperscript{Δ}} mice compared with H\textsuperscript{WT} mice [Supplemental Fig. S2A]. These changes were associated with reduced activation of JNK and reduced phosphorylation of cjun in the liver of H\textsuperscript{JNK\textsuperscript{Δ}} mice compared with H\textsuperscript{WT} mice [Supplemental Fig. S2B]. These observations are consistent with ablation of the JNK signaling pathway in the hepatocytes of H\textsuperscript{JNK\textsuperscript{Δ}} mice. We detected no statistically significant changes in activation of AKT or the ERK and p38 MAPKs in the JNK-deficient liver compared with control liver [Supplemental Fig. S2B].

Measurement of hepatic mass demonstrated that compound deficiency of JNK1 plus JNK2 in hepatocytes caused no significant change in regeneration [Fig. 1B]. To confirm this conclusion, we examined hepatocyte proliferation by measuring the incorporation of bromodeoxyuridine (BrdU) [Fig. 1A]. This analysis demonstrated increased BrdU incorporation at 48 h post-PHx in both H\textsuperscript{WT} and H\textsuperscript{JNK\textsuperscript{Δ}} mice, but the amount of BrdU incorporation was reduced in H\textsuperscript{JNK\textsuperscript{Δ}} mice compared with H\textsuperscript{WT} mice [Fig. 1D]. However, a time-course analysis demonstrated that the overall hepatic proliferation in H\textsuperscript{WT} and H\textsuperscript{JNK\textsuperscript{Δ}} mice following PHx was similar [Fig. 1C]. These data demonstrate that compound JNK deficiency does not prevent hepatic regeneration following PHx.

The discovery that mice with compound JNK deficiency in hepatocytes were capable of hepatic regeneration following PHx was unexpected for two reasons. First, hepatic regeneration is strongly reduced in H\textit{jnk1\textsuperscript{−/−}/jnk2\textsuperscript{−/−}} mice [Supplemental Fig. S1; Hui et al. 2008]. Second, studies of \textit{jnk1\textsuperscript{−/−}/jnk2\textsuperscript{−/−}} primary MEFs demonstrate that these JNK-deficient cells exhibit severe defects in proliferation, including early senescence [Tournier et al. 2000; Das et al. 2007]. Compound JNK deficiency was therefore anticipated to similarly cause growth retardation in other cell types, including hepatocytes. We therefore sought to confirm that liver regeneration following PHx is JNK-independent using a different mouse model with compound hepatic JNK deficiency [Supplemental Fig. S3]. Together, these data confirm the conclusion that hepatic regeneration following PHx occurs in mice with deficiency of both JNK1 and JNK2 in the liver [Fig. 1; Supplemental Fig. S3]. JNK is therefore not essential for hepatic regeneration.

**JNK deficiency in hepatocytes promotes HCC**

The conclusion that JNK is not essential for hepatic regeneration led us to question whether JNK is required for hepatocyte proliferation in the context of HCC. We therefore tested whether JNK in hepatocytes is required for HCC development by treating H\textsuperscript{WT} and H\textsuperscript{JNK\textsuperscript{Δ}} mice with the carcinogen DEN. Previous reports indicate that DEN-induced HCC is markedly reduced in J\textsuperscript{knk1\textsuperscript{−/−}} mice [Sakurai et al. 2006; Hui et al. 2008]. In contrast, we found that H\textsuperscript{JNK\textsuperscript{Δ}} mice developed HCC following exposure to DEN [Fig. 2A, Supplemental Fig. S4]. Indeed, the tumor size in H\textsuperscript{JNK\textsuperscript{Δ}} mice was significantly greater than in H\textsuperscript{WT} mice [Fig. 2B]. However, no significant difference in tumor incidence or the number of tumor nodules between H\textsuperscript{WT} and H\textsuperscript{JNK\textsuperscript{Δ}} mice was detected [Fig. 2C]. This effect of JNK deficiency to increase HCC was associated with increased hepatic expression of cjun [Supplemental Fig. S1; Hui et al. 2008].
Fig. S5A]. Histological analysis of hepatic sections demonstrated the presence of lesions ranging from adenoma to carcinoma in HWT mice, but primarily carcinoma in HΔJNK mice [Fig. 2D, Supplemental Figs. S6, S7]. Immunoblot analysis demonstrated that HΔJNK tumors were JNK deficient [Fig. 2E]. Together, these data indicate that JNK in hepatocytes acts to reduce tumor development in this model of HCC. However, Kaplan-Meier analysis demonstrated that the increased tumor burden in the HΔJNK mice did not cause a statistically significant ($P = 0.055$) change in survival compared with HWT mice [Fig. 2F].

**JNK deficiency can prevent HCC development**

The finding that JNK in hepatocytes acts to reduce HCC (Fig. 2) was not anticipated because studies of Jnk1−/− mice [but not Jnk2−/− mice] have indicated that JNK plays a critical role in HCC development [Sakurai et al. 2006; Hui et al. 2008]. Together, these findings suggest that JNK may both inhibit and promote tumor formation during DEN-induced HCC development. The protumorigenic role of JNK does not require the function of JNK in hepatocytes (Fig. 2), but may involve functions of JNK in nonparenchymal cells. To test this hypothesis, we examined DEN-induced HCC development in mice with compound JNK deficiency using Mx-1-Cre mice and conditional gene ablation in hepatocytes and nonparenchymal cells [Das et al. 2009].

Control MxWT mice [Mx-1-Cre+/−] and JNK-deficient MxΔJNK mice [Mx-1-Cre−/− Jnk1LoxP/LoxP Jnk2−/−] were tested for HCC development. We found that HCC was strongly suppressed in MxΔJNK mice compared with MxWT mice [Fig. 3A, Supplemental Fig. S4]. The tumor size in MxΔJNK mice was significantly smaller than in MxWT mice [Fig. 3B]. Moreover, both the tumor incidence and the number of tumor nodules were reduced in MxΔJNK mice compared with MxWT mice [Fig. 3C]. This effect of JNK deficiency to suppress HCC was associated with markedly reduced hepatic expression of cJun [Supplemental Fig. S5B]. Histological analysis of hepatic tissue sections demonstrated lesions ranging from adenoma to carcinoma in MxWT mice, but primarily only localized adenoma were detected in MxΔJNK mice [Fig. 3D; Supplemental Figs. S7, S8]. Immunoblot analysis demonstrated that MxΔJNK tumors were JNK-deficient (Fig. 3E). Kaplan-Meier analysis demonstrated that the reduced tumor burden in MxΔJNK mice caused significantly increased survival ($P = 0.017$) compared with MxWT mice [Fig. 3F]. Together, these data demonstrate that JNK can play a protumorigenic role in HCC development. This protumorigenic role does not require JNK in hepatocytes (Fig. 2), but does require JNK in nonparenchymal cells [Fig. 3].

**JNK regulation of HCC proliferation mediated by p21CIP1 and cMyc**

JNK-mediated transcriptional down-regulation of the growth inhibitor p21CIP1 and up-regulation of the growth promoter cMyc have been implicated as critical steps during HCC development [Hui et al. 2008]. We therefore examined p21CIP1 and cMyc expression in the liver of mice with compound deficiency of JNK1 plus JNK2 [Fig. 4]. We found that p21CIP1 expression was significantly increased in DEN-treated JNK-deficient mice [HΔJNK and
Mx\(^{\Delta JNK}\) compared with DEN-treated control mice [H\(^{WT}\) and Mx\(^{WT}\)] (Fig. 4). Since HCC is increased in H\(^{\Delta JNK}\) mice (Fig. 2) and decreased in Mx\(^{\Delta JNK}\) mice [Fig. 3], these data demonstrate no correlation between \(p21^{Cip1}\) expression and HCC development in compound JNK-deficient mice. In contrast, an association between JNK, expression of cMyc, and Cyclin D1 mRNA was examined by RT–PCR using TaqMan assays. The data presented are normalized for the amount of Gapdh mRNA in each sample and represent the mean ± SD (\(n = 5\)). Statistically significant differences are indicated. (*\( P < 0.05\). A) Control mice [H\(^{WT}\)] and mice with JNK deficiency in hepatocytes [H\(^{\Delta JNK}\)]. (B) Control mice [Mx\(^{WT}\)] and mice with JNK deficiency in hepatocytes and nonparenchymal cells [Mx\(^{\Delta JNK}\)].

\(JNK\) regulates compensatory proliferation during HCC development

It is established that compensatory hepatocyte proliferation plays a major role in the development of HCC (Fausto 1999). This form of hepatocyte proliferation represents a regenerative response to hepatocyte death that is associated with inflammation (Grivennikov et al. 2010). We therefore examined hepatocyte proliferation and death in control mice [H\(^{WT}\) and Mx\(^{WT}\)] and mice with compound deficiency of JNK1 plus JNK2 [H\(^{\Delta JNK}\) and Mx\(^{\Delta JNK}\)] following treatment with DEN. Sections of liver tumors were stained for the proliferation marker PCNA [Fig. 5A,B] and for cell death using the TUNEL assay [Fig. 5C,D]. We also examined caspase activation by immunoblot analysis of Caspase 3 processing to the activated form [Supplemental Fig. S9]. Studies of mice with hepatocyte-specific compound JNK deficiency [H\(^{\Delta JNK}\) mice] demonstrated increased cell death and increased proliferation compared with control H\(^{WT}\) mice [Fig. 5A,C; Supplemental Fig. S9]. In contrast, compound deficiency of JNK in hepatocytes plus nonparenchymal cells [Mx\(^{\Delta JNK}\) mice] caused both reduced cell death and reduced proliferation compared with control Mx\(^{WT}\) mice [Fig. 5B,D; Supplemental Fig. S9]. These data suggest that decreased compensatory proliferation may account for the reduction in tumor growth caused by JNK in hepatocytes, and that increased compensatory proliferation may account for the protumorigenic activity of JNK in nonparenchymal cells.

\(JNK\) regulates the expression of hepatic cytokines

The compensatory proliferation caused by hepatocyte death [Fausto 1999] may be mediated by the release of IL1α by necrotic cells (Chen et al. 2007; Sakurai et al. 2008) and the subsequent expression of protumorigenic IL6 (and also TNFα) by hepatic innate immune cells (Naugler et al. 2007; Park et al. 2010). Indeed, HCC development in mice can be suppressed by ablation of the Il6 and Tifa genes (Naugler et al. 2007; Park et al. 2010). We therefore examined the expression of inflammatory cytokines in control and JNK-deficient mice [Fig. 6]. Studies of mice with compound deficiency of JNK1 plus JNK2 in hepatocytes [H\(^{\Delta JNK}\) mice] demonstrated that JNK deficiency caused increased hepatic expression of Il1α, Il1β, Il6, and Tgfβ1 mRNA [Fig. 6A; Supplemental Fig. S10]. In contrast, studies of mice with compound deficiency of JNK1 and JNK2 in hepatocytes plus nonparenchymal cells [Mx\(^{\Delta JNK}\) mice] demonstrated that JNK deficiency caused decreased expression of Il1α, Il1β, Il6, Tgfβ1, and Tifa mRNA [Fig. 6B; Supplemental Fig. S10].

Together, these data indicate that JNK deficiency in hepatocytes [H\(^{\Delta JNK}\) mice] causes increased cell death [Fig. 5C; Supplemental Fig. S9], increased expression of inflammatory cytokines [Figs. 6A; Supplemental Fig. S10], and increased compensatory hepatocyte proliferation [Fig. 5A]. However, JNK deficiency in hepatocytes plus nonparenchymal cells [Mx\(^{\Delta JNK}\) mice] causes decreased cell death [Fig. 5D; Supplemental Fig. S9], decreased expression of inflammatory cytokines [Figs. 6B; Supplemental Fig. S10], and decreased compensatory hepatocyte proliferation [Fig. 5B]. The primary actions of JNK disrupted in H\(^{\Delta JNK}\) mice may reflect a role for JNK in cell survival (Lamb et al. 2003) and thus decreased compensatory proliferation. The primary actions of JNK disrupted in
MxΔJNK mice may be expression of the protumorigenic cytokines IL6 and TNFα [Sabio et al. 2008; Das et al. 2009].

The role of IL6 in JNK-regulated development of HCC

It is established that IL6 plays a key role in hepatocyte proliferation [Cressman et al. 1996], and that IL6 is critically required for DEN-induced HCC [Naugler et al. 2007]. We therefore examined the hepatic IL6 signaling pathway in mice with compound JNK deficiency. Treatment of control mice with DEN caused an increase in hepatic IL6 mRNA and IL6 protein in the blood (Fig. 6). The Socs3 gene is a target of IL6-stimulated STAT3 signaling in the liver. Treatment of mice with DEN caused increased Socs3 mRNA expression (Fig. 6). This DEN-induced increase in Socs3 gene expression was suppressed in MxΔJNK mice (Fig. 6B) and potentiated in H^ΔJNK mice [Fig. 6A]. These data are consistent with the increased HCC and hepatic IL6 expression in H^ΔJNK mice (Figs. 2, 6A) and reduced HCC and hepatic IL6 expression in MxΔJNK mice (Figs. 3, 6B).

The mir-21 gene has been identified as a target of inflammatory signaling pathways, including IL6 [Jazbutyte and Thum 2010]. Indeed, treatment of mice with IL6 caused increased hepatic expression of mir-21 (Supplemental Fig. S11), and it is established that IL6-induced mir-21 expression is mediated by two conserved STAT3-binding sites in the mir-21 promoter [Loffler et al. 2007]. The expression of mir-21 in the liver is increased during HCC development [Kutay et al. 2006; Meng et al. 2007; Connolly et al. 2008; Jiang et al. 2008], and studies using a knockdown approach demonstrate that mir-21 contributes to HCC proliferation [Connolly et al. 2008]. These data establish that mir-21 plays an important role in HCC development.

We examined the effect of JNK deficiency on hepatic expression of mir-21. Control studies demonstrated that DEN caused increased expression of mir-21 [Supplemental Fig. S12]. Hepatocyte-specific JNK deficiency [H^ΔJNK mice] caused increased mir-21 expression compared with control [H^WT] mice following treatment with DEN [Supplemental Fig. S13A]. In contrast, DEN-treated mice with JNK deficiency in hepatocytes and nonparenchymal cells [MxΔJNK] expressed reduced mir-21 compared with control [MxWT] DEN-treated mice [Supplemental Fig. S13A]. These effects of JNK deficiency on mir-21 expression are consistent with the observed increase [H^ΔJNK] and decrease [MxΔJNK] in the expression of hepatic IL6 [and other inflammatory cytokines] following DEN treatment [Fig. 6; Supplemental Fig. S10].

Targets of mir-21 include repressors of the AKT [Pten] and receptor tyrosine kinase [Sprouty] signaling pathways [Cabrita and Christofori 2008]. We therefore examined Pten and Sprouty expression in DEN-treated control [MxWT] mice and JNK-deficient [MxΔJNK] mice. JNK deficiency did not change the expression of Pten or Sprouty mRNA [Supplemental Fig. S13C,D]. Similarly, JNK deficiency did not cause altered expression of Pten protein [Supplemental Fig. S13D]. In contrast, JNK deficiency caused increased expression of Sprouty protein [Supplemental Fig. S13C]. To test whether this increased expression of Sprouty is functionally relevant, we examined signaling pathways downstream from activated receptor tyrosine kinases, including AKT and ERK. We found that increased expression of the negative regulator Sprouty in JNK-deficient [MxΔJNK] mice was associated with reduced AKT and ERK activity [Supplemental Fig. S13B]. These data suggest that Sprouty [Supplemental Fig. S13C] may be a relevant target of the mir-21 pathway that is required for HCC development [Connolly et al. 2008].
Discussion

The role of JNK in proliferation is cell type-dependent

It is established that JNK is critically required for the proliferation of primary MEFs (Davis 2000). Compound mutant Jnk1−/− Jnk2−/− MEFs exhibit a severe growth retardation phenotype (Tournier et al. 2000) and premature senescence (Das et al. 2007). The rapid senescence of primary Jnk1−/− Jnk2−/− MEFs is mediated by increased expression of the Trp53 tumor suppressor (Das et al. 2007). This engagement of p53-dependent senescence in primary Jnk1−/− Jnk2−/− MEFs (Das et al. 2007) is similar to cjun−/− MEFs (Schreiber et al. 1999). Indeed, reduced expression of cjun by Jnk1−/− Jnk2−/− MEFs may contribute to the early senescence phenotype (Ventura et al. 2003). These observations indicate that JNK may have a general role in the proliferation of many cell types. Reports that JNK1 plays an essential role in both hepatic regeneration following PHx (Hui et al. 2008) and DEN-induced HCC (Sakurai et al. 2006; Hui et al. 2008) are consistent with this conclusion.

The finding that compound JNK deficiency does not prevent hepatocyte proliferation was unexpected (Figs. 1, 2; Supplemental Fig. S3). The simplest explanation for these data is that JNK is not required for proliferation of hepatocytes (Fig. 1; Supplemental Fig. S3), but JNK is
selectively required to prevent Trp53-dependent senescence of MEFs [Das et al. 2007]. Cell type-specific responses to JNK deficiency may reflect differences in the requirement of JNK for the expression of cjun, a repressor of the Trp53 promoter [Schreiber et al. 1999]. Compound JNK deficiency in MEFs causes down-regulation of cjun expression [Ventura et al. 2003]. In contrast, compound JNK deficiency in hepatocytes did not cause significantly decreased cjun expression [Supplemental Fig. S2A]. The failure of JNK deficiency to down-regulate cjun expression in hepatocytes may reflect signaling pathway redundancy, including roles of JNK in AP1-dependent cjun expression [Ventura et al. 2003] and p38 MAPK in MEF2-dependent cjun expression [Han et al. 1997]. Indeed, it is intriguing that the p38 MAPK pathway in hepatocytes is constitutively activated and subject to metabolic regulation [Mendelson et al. 1996], but p38 MAPK is stress-inducible from a low basal activity in MEFs [Ringeaude et al. 1995]. The p38 MAPK pathway may therefore act redundantly with JNK to regulate cjun expression in hepatocytes, but the p38 MAPK pathway may not compensate for JNK deficiency in MEFs. Cross-talk, partially redundant signaling, and partially antagonistic signaling by the JNK and p38 MAPK pathways may therefore contribute to the tissue-specific effects of JNK on proliferation [Wagner and Nebreda 2009].

The role of JNK in liver regeneration

The function of JNK in hepatocytes and nonparenchymal cells of the liver is not required for hepatic regeneration following PHx [Fig. 1; Supplemental Fig. S3]. Nevertheless, liver regeneration is reduced in Jnk1−/− mice [Supplemental Fig. S1; Hui et al. 2008]. The mechanism that accounts for this phenotype of Jnk1−/− mice is unclear. The simplest hypothesis is that JNK1 plays a critical role in a nonhepatic tissue, and that this JNK1-dependent function is disrupted in Jnk1−/− mice. The identity of the relevant tissue that mediates JNK1-dependent hepatic regeneration has not been established. One possibility is represented by the hypothalamus, a region of the brain that strongly influences liver regeneration [Kiba 2002]. This possibility is consistent with the discovery that the major metabolic phenotypes of Jnk1−/− mice are largely accounted for by the effects of JNK1 deficiency in the hypothalamus, rather than in peripheral tissues [Belgardt et al. 2010; Sabio et al. 2010]. A goal for further studies will be to identify the specific tissue that accounts for the required role of JNK1 for hepatic regeneration. This role of JNK1 contrasts with the absence of a requirement for hepatic JNK during liver regeneration following PHx [Fig. 1; Supplemental Fig. S3].

The role of JNK in the promotion of HCC

It is established that JNK plays a key role in the development of DEN-induced HCC [Sakurai et al. 2006; Hui et al. 2008]. Two mechanisms have been reported to account for JNK-mediated protumorigenic activity. First, JNK1 may be required for down-regulation of p21CIP1 expression and up-regulation of cMyc expression [Hui et al. 2008]. Our analysis demonstrates that p21CIP1 expression does not correlate with tumorigenic potential in studies of HCC development in compound JNK-deficient mice [H JNK and MxαJNK] [Fig. 4]. In contrast, expression of cMyc [Fig. 4] correlates with the tumor burden in H JNK and MxαJNK mice [Figs. 2, 3]. These data are consistent with a role for cMyc in JNK-dependent formation of HCC [Hui et al. 2008].

A second proposed mechanism is that JNK1 causes hepatocyte death [Sakurai et al. 2006], resulting in compensatory proliferation that contributes to the development of HCC [Fausto 1999]. However, compound deficiency of JNK1 and JNK2 in hepatocytes [H JNK mice] does not inhibit hepatocyte death following treatment with DEN [Fig. 5C, Supplemental Fig. S9]. These data demonstrate that JNK-mediated hepatocyte death does not contribute to compensatory proliferation and HCC. This analysis indicates that another mechanism must account for the protumorigenic effects of JNK on HCC development.

The observation that compound JNK deficiency in hepatocytes [H JNK mice] does not reduce DEN-induced HCC [Fig. 2], but compound JNK deficiency in hepatocytes plus nonparenchymal cells [MxαJNK mice] does reduce DEN-induced HCC [Fig. 3], provides important insight into the mechanism of JNK-promoted HCC development. These findings indicate that the protumorigenic function of JNK may be localized to nonparenchymal cells, rather than hepatocytes. Indeed, functions of nonparenchymal cells are implicated in HCC development [Maeda et al. 2005; Grivennikov et al. 2010]. Specifically, the release of IL1α by necrotic cells [Chen et al. 2007; Sakurai et al. 2008], and the subsequent expression of the protumorigenic cytokine IL6 [and also TNFα in obese mice] by hepatic innate immune cells, including Kupffer cells [Naugler et al. 2007; Park et al. 2010], may drive compensatory hepatocyte proliferation and, subsequently, HCC [Fig. 7]. It is therefore significant that compound JNK deficiency in MxαJNK mice, but not H JNK mice, causes markedly decreased expression of IL6 and TNFα [Fig. 6]. These data are consistent with the conclusion that JNK is required for the expression of the protumorigenic cytokines IL6 and TNFα by hepatic innate immune cells during the development of HCC [Fig. 6B]. Thus, the protumorigenic activity of JNK may be mediated by a role for JNK in nonparenchymal cells [Fig. 3] in the creation of an inflammatory environment that supports HCC development [Grivennikov et al. 2010]. The previously described genetic interactions between Jnk1 and p21CIP1 [Hui et al. 2008] may therefore reflect an inhibitory action of p21CIP1 on hepatic innate immune cells, rather than hepatocytes. Studies using mice with tissue-specific p21CIP1 deficiency will be required to resolve this question.

The role of JNK in hepatocytes during HCC development

Studies of mice with compound deficiency of JNK1 plus JNK2 demonstrate that cell death and compensatory

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proliferation are key targets of JNK function in HCC [Fig. 5]. Specifically, we find that compound JNK deficiency in hepatocytes causes increased hepatocyte death and, consequently, increased compensatory proliferation and HCC development [Figs. 2, 5]. The increased death caused by compound JNK deficiency is consistent with previous studies that demonstrate a role for JNK in cell survival [Kuan et al. 1999; Lamb et al. 2003] and the presence of JNK-independent pathways that can mediate hepatocyte death [Das et al. 2009]. Although the cell type of HCC origin is unclear [Mishra et al. 2009], tumors detected using Albumin-Cre transgenic mouse models of DEN-induced HCC demonstrate efficient ablation of floxed genes in tumor cells [Fig. 2E; Maeda et al. 2005]. These data suggest that JNK in hepatic parenchymal cells functions to reduce tumor development in the DEN model of HCC. This effect of JNK to influence hepatocyte death, compensatory proliferation, and HCC development [Figs. 2, 5] is similar to the reported effects of hepatocyte-specific deficiency of the NF-κB activator IKKβ [Maeda et al. 2005]. Together, these data are consistent with the proposed role of compensatory proliferation in hepatic tumor development [Fausto 1999].

Conclusions

The results of this study demonstrate that JNK plays a complex role in the development of HCC. JNK is required for HCC development because it functions in nonparenchymal cells to cause expression of protumorigenic cytokines, including IL6 and TNFα. In contrast, JNK in hepatocytes reduces tumor development by decreasing hepatocyte death and compensatory proliferation. It is likely that previous studies using mice with whole-body knockout of Jnk1 or Jnk2 reflect a composite phenotype derived from the cell type-specific functions of JNK in hepatocytes and nonparenchymal cells.

We conclude that JNK both promotes and inhibits tumor development in the DEN model of HCC. This conclusion has implications for the more general role of JNK in cancer. Both tumor promotion and inhibition by JNK may contribute to cancer development [Davis 2000, Whitmash and Davis 2007]. This complicates the analysis of JNK pathway mutations that have been identified in human cancer [Greenman et al. 2007; Kan et al. 2010]. Moreover, this dual role of JNK should be considered in the context of the potential use of JNK as a therapeutic target for drug development and the treatment of human cancer.

Materials and methods

Genotype analysis

Genotype analysis was performed by PCR using genomic DNA as the template. The Jnk1LoxP/LoxP mice [Das et al. 2009] and Jnk2−/− mice [Yang et al. 1998], B6.Cg-Tg(Alb-cre)21Mgn/J mice [Postic et al. 1999], B6.Cg-Tg(Mx1-cre)1Cgn/J mice [Kuhn et al. 1995], and C57BL/6j mice were obtained from The Jackson Laboratories [stock numbers 003574, 003556, and 000664, respectively]. The mutant mice were maintained on a C57BL/6j strain background [backcrossed 10 generations], and were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). Deletion of floxed alleles in Mx1-Cre mice was performed by treatment of 4-wk-old mice with 20 μg/g polyinosinic-polycytidylic acid [polyIC] [Mikkola et al. 2003] followed by recovery [4 wk]. Control mice were similarly injected with polyIC. PHx was performed on 10-wk-old mice using methods described previously [Greene and Puder 2003]. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.

HCC assays

Carcinogen-induced HCC was studied using procedures described previously [Sakurai et al. 2008]. Two-week-old male mice were treated by intraperitoneal (i.p.) injection with a single dose [25 mg/kg] of DEN [Sigma, N0258] diluted in glycercyl trioctanoate [Sigma, T9126]. Control mice were injected with solvent alone. The mice were euthanized at 38 wk of age to examine tumor development. Surface tumor size and number were measured using stereomicroscopy [Maeda et al. 2005]. The Cre+ mice were detected using the amplimers 5′-AGGAGTTATGCCCTCTCGTGTCC-T3′ and 5′-GAACCACGTGTTCCAATTTCCATCC-3′. The Jnk1LoxP/Jnk1LoxP mice were measured using stereomicroscopy (Maeda et al. 2005). We previously described Jnk1LoxP/LoxP mice [Das et al. 2009] and Jnk2−/− mice [Yang et al. 1998], B6.Cg-Tg(Alb-cre)21Mgn/J mice [Postic et al. 1999], B6.Cg-Tg(Mx1-cre)1Cgn/J mice [Kuhn et al. 1995], and C57BL/6j mice were obtained from The Jackson Laboratories [stock numbers 003574, 003556, and 000664, respectively]. The mutant mice were maintained on a C57BL/6j strain background [backcrossed 10 generations], and were housed in a facility accredited by the American Association for Laboratory Animal Care (AALAC). Deletion of floxed alleles in Mx1-Cre mice was performed by treatment of 4-wk-old mice with 20 μg/g polyinosinic-polycytidylic acid [polyIC] [Mikkola et al. 2003] followed by recovery [4 wk]. Control mice were similarly injected with polyIC. PHx was performed on 10-wk-old mice using methods described previously [Greene and Puder 2003]. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School.
Mx1-Cre<sup>−/−</sup> mice with JNK-deficient Mx1-Cre<sup>+/−</sup> mice or control Alb-Cre<sup>−/−</sup> mice with JNK-deficient Alb-Cre<sup>+/−</sup> mice.

**Serum analysis**

Alanine transaminase [ALT] and aspartate aminotransferase [AST] activity in serum was measured using the ALT and AST Reagent kit [Pointe Scientific] with a Tecan Sapphire microplate reader [Tecan Trading AG]. The serum concentration of cytokines was measured by multiplexed ELISA using a Luminex 200 instrument [Millipore].

**Biochemical analysis**

Immunoblot analysis was performed by probing with antibodies to activated Caspase 3 [Cell Signaling, #9661] Sprouty2 [Abcam, #ab50317], PTEN [Cell Signaling, #9198], p21 [Santa Cruz Biotechnology, #SC 6246], and α-Tubulin [Sigma, #T5168]. Immunoblots were quantitated using a LiCOR Odyssey imager. The amount of total and phospho-JNK, ERK, p38 MAPK, and AKT in liver sections were determined (12 h) at 4°C with a biotin-linked antibody to PCNA.

Analysis of liver sections

Histology was performed using tissue fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Sections [7 μm] were cut and stained using hematoxylin and eosin [American Master Tech Scientific]. Terminal deoxynucleotidyltransferase-mediated nick end labeling [TUNEL] staining of deparaffinized liver sections was performed using the In Situ Cell Death Detection kit Fluorescein [Roche] following antigen retrieval [Antigen Unmasking Solution, Vector Laboratories]. Immunofluorescence analysis of deparaffinized liver sections was performed using antigen retrieval [Vector Laboratories]; incubation [1 h] with a 3% bovine serum albumin (BSA) and 0.4% Triton X-100 in phosphate-buffered saline (PBS); incubation [12 h] at 4°C with a biotin-linked antibody to PCNA [Zymed Laboratories, 13-3940] in PBS with 3% BSA, and detection of immune complexes with Streptavidin/Biotin-based Link-Label Detection kit [Biogenex, Super-sensitive Link label immunohistochemistry detection system] and DAB substrate [Vector Laboratories, #SK4100].

**Statistical analysis**

Differences between groups were examined for statistical significance using the Student's t-test or analysis of variance (ANOVA) with the Fisher's test. Kaplan-Meier analysis was performed using the log-rank test.

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**References**


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