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c-Met activation through a novel pathway involving osteopontin mediates oncogenesis by the transcription factor LSF

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Abstract

Background and Aims—Understanding the molecular pathogenesis of hepatocellular carcinoma (HCC) would facilitate development of targeted and effective therapies for this fatal disease. We recently demonstrated that the cellular transcription factor Late SV40 Factor (LSF) is overexpressed in more than 90% of human HCC cases, compared to normal liver, and plays a seminal role in hepatocarcinogenesis. LSF transcriptionally upregulates osteopontin (OPN) that plays a significant role in mediating the oncogenic function of LSF. The present study aims at a better understanding of LSF function by analyzing the signaling pathway modulated by LSF.

Methods—Phospho-receptor tyrosine kinase (RTK) array was performed to identify which receptor tyrosine kinases are activated by LSF. Immunohistochemical analysis using tissue microarray was performed to establish correlation among LSF, OPN and phospho-c-Met levels in HCC patients. Co-immunoprecipitation analysis was performed to check OPN-induced CD44 and c-Met interaction. Inhibition studies using chemicals and siRNAs were performed in vitro and in vivo using nude mice xenograft models to establish the importance of c-Met activation in mediating LSF function.

Results—Secreted OPN, induced by LSF, activates c-Met via a potential interaction between OPN and its cell surface receptor CD44. A significant correlation was observed among LSF, OPN and activated c-Met levels in HCC patients. Chemical or genetic inhibition of c-Met resulted in profound abrogation of LSF-mediated tumorigenesis and metastasis in nude mice xenograft studies.

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Conclusions—The present findings elucidate a novel pathway of c-Met activation during hepatocarcinogenesis and support the rationale of using c-Met inhibitors as potential HCC therapeutics.

Keywords

Late SV40 Factor; CD44; Hepatocellular carcinoma; c-Met; osteopontin

Introduction

Hepatocellular carcinoma (HCC) is a major global health problem [1]. The incidence of HCC is increasing in the West, and it is now the third highest cause of cancer-related death globally [1]. In most cases HCC is diagnosed at an advanced stage and has a dismal prognosis owing to the inherent resistance of the disease to conventional anti-cancer therapies [2]. No systemic therapy has improved survival in patients with advanced HCC. In these contexts, defining the molecular determinants of HCC pathogenesis is necessary to develop rationally based targeted and potentially effective therapies for this fatal disease. Our efforts to identify novel regulators of hepatocarcinogenesis revealed overexpression of the transcription factor Late SV40 Factor (LSF) in human HCC cell lines and in more than 90% cases of human HCC, compared to normal hepatocytes and liver, respectively [3]. LSF expression level correlated with the stages and grades of HCC indicating that LSF might be a new prognostic marker for HCC. Stable overexpression of LSF induced a highly aggressive, angiogenic and multi-organ metastatic phenotype in the non-tumorigenic human HCC cell line HepG3 [3]. Conversely, stable dominant-negative inhibition of LSF profoundly abrogated tumor growth and metastasis by a highly aggressive human HCC cell line QGY-7703 [3]. Microarray analysis identified a plethora of genes modulated by LSF including those associated with proliferation, invasion, angiogenesis, metastasis and chemoresistance [3]. Osteopontin (OPN), which is implicated in augmenting every step in tumor progression and metastasis, was found to be the most robustly LSF-induced gene in HCC [3, 4]. We demonstrated that LSF transcriptionally upregulated OPN by directly binding to its promoter and stable shRNA-mediated knockdown of OPN profoundly inhibited oncogenic properties of LSF [3]. We also documented that by transcriptionally regulating thymidylate synthase, LSF contributes to 5-fluorouracil resistance [5]. Thus LSF contributes to an important hallmark of aggressive cancers, i.e., chemoresistance.

c-Met receptor tyrosine kinase (RTK), a cell surface receptor for hepatocyte growth factor (HGF), conveys a unique combination of pro-migratory, anti-apoptotic and mitogenic signals [6-8]. When activated, c-Met initiates epithelial-mesenchymal transition (EMT) by facilitating cell scattering, thereby facilitating migration and invasive growth [7]. c-Met activation has been documented in a variety of cancers and can be induced by gene amplification, transcriptional upregulation, ligand-dependent autocrine or paracrine mechanisms, mutational activation or cross-talk with other RTKs [6, 9]. c-Met plays a pivotal role in HCC that was confirmed by the observation that conditional overexpression of wild-type c-Met in hepatocytes of transgenic mice (an experimental condition that mimics the spontaneous amplification of the c-Met gene observed in human tumors) is sufficient to cause hepatocellular carcinoma that regress following transgene inactivation [10]. Binding of HGF to c-Met leads to autophosphorylation of c-Met in Y1234 and Y1235 residues in the catalytic domain followed by phosphorylation at Y1349 and Y1356 that is essential for recruitment of adaptor proteins [6]. In the absence of ligand, c-Met can be activated by integrin-interaction during cell adhesion, the hyaluronan receptor CD44 and interaction with other RTKs such as EGFR or Ron kinase [6, 11, 12]. c-Met activation leads to activation of several pro-survival signaling pathways, such as MEK/ERK and PI3K/Akt pathways, and molecules important for migration, invasion and metastasis, such as Src, STAT3 and the

Rho-like GTPase Rac1 [6, 9]. Inhibition of c-Met by blocking antibodies or small molecule inhibitors are being evaluated in Phase I/II clinical trials as potential anti-cancer strategies in diverse cancers [13].

In the present study we document that LSF overexpression activates c-Met. In experiments employing 'gain-of-function' and 'loss-of-function' approaches we document that secreted OPN, induced by LSF, activates c-Met by interaction with CD44. c-Met activation is an important contributor to LSF-mediated oncogenesis. Since LSF is overexpressed in more than 90% of human HCC, c-Met inhibition might be an essential component of hepatocarcinogenesis and may represent a viable target for developing a combinatorial therapeutic strategy for HCC.

Materials and Methods

Cell lines and culture condition

Human HCC cell lines HepG3 and QGY-7703 were cultured as described [14]. HepG3 clones stably expressing LSF (LSF-1 and LSF-17) and QGY-7703 clones stably expressing LSFdn (QGY-LSFdn-8 and QGY-LSFdn-15) were generated as described [3]. LSF-17-Consh-15, LSF-17-OPNsh-6 and LSF-17-OPNsh-18 clones were generated as described [3]. The LSF-17 clone of HepG3 cells was transduced with a pool of three to five lentiviral vector plasmids, each encoding target-specific 19-25 nt (plus hairpin) shRNAs designed to knockdown c-Met gene expression (Santa Cruz Biotechnology). Individual colonies were selected by puromycin for two weeks to generate LSF-17-shMet-1 and LSF-17-shMet-17 clones.

Tissue microarray and human HCC samples

Human HCC tissue microarray (Imgenex; IMH-360) containing 40 primary HCC, 10 metastatic HCC and 9 normal adjacent liver samples was obtained from Imgenex Corp. Patient samples were obtained from the Liver Tissue Cell Distribution System (LTCDS), a National Institutes of Health (NIH) service contract to provide human liver and isolated hepatocytes from regional centers for distribution to scientific investigators throughout the United States (NIH contract N01-DK-7-0004/HHSN267200700004C). The 18 matched normal liver and HCC sample include LTCDS # 1100, 1107, 1135, 1143, 1153, 1154, 1164, 1169, 1172, 1174, 1194, 1216, 1237, 1246, 1260, 1264, 1276 and 1282.

Results

LSF activates c-Met

HepG3 is a human HCC cell line that is poorly aggressive, non-tumorigenic in nude mice and expresses low level of LSF [14]. In contrast, QGY-7703 is a highly aggressive, metastatic human HCC cell line that expresses high levels of LSF [14]. We established stable cell lines overexpressing LSF in HepG3 background (LSF-1 and LSF-17) and documented that these clones form highly aggressive, multi-organ metastatic tumors in nude mice [3]. Conversely, we established stable cell lines expressing an LSF dominant negative mutant in a QGY-7703 background (LSFdn-8 and LSFdn-15) that resulted in profound inhibition in tumor growth and metastasis in nude mice when compared to control clones [3].

Since LSF modulates multiple signaling pathways in cells, profoundly modulates global gene expression profiles and induces marked phenotypic changes in HCC cells, we hypothesized that LSF might activate receptor tyrosine kinases (RTKs) that induce these profound changes. For this purpose, we used a human phospho-RTK array kit to detect

relative levels of tyrosine phosphorylation of human RTKs induced by LSF. We used lysates from Control-8 (control neomycin-resistant clone) and LSF-17 clones of HepG3 cells for this assay. A robust increase in c-Met, a moderate increase in Ret and a small increase in ErbB3 and EGFR activation were observed in the LSF-17 clone as compared to the Control-8 clone (Fig. 1A). Since c-Met showed the most profound activation we investigated the molecular mechanism underlying this phenomenon. The level of activated (phosphorylated) c-Met was significantly higher in LSF-1 and LSF-17 clones compared to the Control-8 clone of HepG3 cells. Similarly, activated c-Met was significantly downregulated in LSFdn-8 and LSFdn-15 clones, compared to the Control-7 clone of QGY-7703 cells (Fig. 1B). The level of total c-Met did not change in either group. We compared LSF and phospho-c-Met levels in normal liver and matched HCC from 18 patients by immunohistochemical analysis. Thirteen of these patients showed increased expression of LSF as well as phospho-c-Met in HCC compared to normal liver. A representative result from one patient is shown in Fig. 1C. These findings were extended by immunohistochemistry in a tissue microarray containing 50 human HCC sample. A statistically significant correlation ($R=0.7148$) was observed between LSF and phospho-c-Met levels in these patients establishing the link between LSF and c-Met activation in the clinical situation (Fig. 1D).

OPN mediates LSF-induced c-Met activation

The observation that total c-Met level did not change either by LSF overexpression or inhibition indicates that induction of transcription of c-Met by LSF may not be the probable mechanism of activation of c-Met. We, therefore, explored alternative mechanism(s) of c-Met activation by LSF. Since the most common mechanism of c-Met activation is binding of its ligand HGF, we reasoned that LSF might induce high HGF levels resulting in c-Met activation. However, LSF expression did not result in increased HGF expression level (Fig. S1) indicating that c-Met activation by LSF occurred independent of HGF. LSF robustly induced the expression of OPN at the transcriptional level and we hypothesized that secreted OPN might result in c-Met activation. Treatment of HepG3 cells with purified OPN resulted in temporal activation of c-Met, which reached the peak level at 30 min post-treatment and then gradually declined although even at 6 h post-treatment the activated c-Met level remained higher than basal level (Fig. 2A). Total c-Met level did not change upon OPN treatment. We compared OPN and phospho-c-Met levels in normal liver and matched HCC from 18 patients by immunohistochemical analysis. Thirteen of these patients showed increased expression of OPN as well as phospho-c-Met in HCC compared to normal liver. A representative result from one patient is shown in Fig. 2B. Statistically significant correlation ($R=0.7057$) was observed between OPN and phospho-c-Met levels by immunohistochemistry of a tissue microarray of 50 human HCC samples, thereby confirming the link between OPN and c-Met activation in a clinical context (Fig. 2C).

To further confirm the involvement of OPN in mediating LSF-induced c-Met activation, we analyzed activated c-Met levels in stable clones of LSF-17 cells expressing OPN shRNA. These clones have been extensively characterized by us previously [3]. Compared to LSF-17-Consh-15 clone (LSF-17 clone stably expressing control scrambled shRNA), c-Met activation was significantly downregulated in LSF-17-OPNsh-6 and LSF-17-OPNsh-18 clones without affecting total c-Met level (Fig. 2D). OPN works through $\alpha v \beta 3$ integrin and CD44 receptors. We blocked these receptors using neutralizing antibodies and analyzed c-Met activation in the LSF-17 clone of HepG3 cells. Blockage of CD44, but not $\alpha v \beta 3$ integrin, inhibited c-Met activation indicating that OPN-CD44 interactions mediate c-Met activation (Fig. 2D). CD44 blocking also resulted in a decrease in total c-Met level indicating that inhibition of CD44 and c-Met interaction might result in increased internalization and degradation of c-Met.

OPN binding to CD44 mediates c-Met activation

We next explored the molecular mechanism of c-Met activation by OPN using co-immunoprecipitation analysis. Using lysates from HepG3 cells cultured in the absence of serum, anti-c-Met antibody could effectively immunoprecipitate both c-Met and CD44, but not $\alpha\beta3$ integrin (Fig. 3A, left panel). In serum-containing culture conditions, anti-c-Met antibody immunoprecipitated CD44 and addition of OPN (100 ng/ml) did not affect the binding affinity between c-Met and CD44 (Fig. 3A, right panel). Conversely, under serum-free conditions in HepG3 cells anti-CD44 antibody effectively pulled down both CD44 and c-Met (Fig. 3B, left panel). In serum-containing conditions, the presence or absence of OPN did not affect the binding affinity between CD44 and c-Met. We reasoned that c-Met and CD44 might interact with each other, however, binding of OPN to CD44 might induce a conformational change leading to increased phosphorylation and activation of c-Met. Indeed, under serum-free conditions, treatment of HepG3 cells with OPN significantly increased the association between CD44 and phospho-c-Met (Fig. 3C). These findings were confirmed using LSF-17 clone of HepG3 cells and QGY-7703 cells (that endogenously express high LSF). Anti-c-Met antibody immunoprecipitated both c-Met and CD44, and not $\alpha\beta3$ integrin, in both cells (Fig. 3D). Conversely, anti-CD44 antibody immunoprecipitated both c-Met and phospho-c-Met (Fig. 3E). These findings indicate that binding of OPN to CD44 might lead to phosphorylation and activation of c-Met.

To confirm the involvement of CD44 in mediating LSF function, we treated LSF-17 cells with anti-CD44 blocking antibody and analyzed Matrigel invasion. Anti-CD44 antibody resulted in significant inhibition of invasion by LSF-17 cells in comparison to the isotype control antibody indicating that CD44 is involved in mediating functional activity of LSF (Fig. 4A).

c-Met plays an important role in mediating LSF function

We next investigated the importance of c-Met activation in mediating LSF function. LSF-17 cells and QGY-7703 cells were treated with the chemical inhibitor of c-Met, SU11274 (5 μ M), and cell viability and Matrigel invasion was monitored. In both cell lines, cell viability, measured by standard MTT assay, was significantly decreased by SU11274 (Fig. 4B and 4C, respectively). Similarly, Matrigel invasion by both LSF-17 and QGY-7703 cells was significantly inhibited by SU11274 (Fig. 4D and 4E, respectively). For invasion assay cells were treated with SU11274 for 24 h, a time point when no significant effect on cell viability was observed. Removal of SU11274 during the invasion assay significantly rescued the cells from inhibition of invasion indicating that persistent inhibition of c-Met activation is obligatory to block LSF-induced augmentation of invasion (Fig. 4D and 4E, respectively). It should be noted that SU11274 treatment did not affect the viability of the parental HepG3 cells that express low levels of phospho-c-Met (data not shown).

To confirm the link between OPN and c-Met, HepG3 cells were treated with OPN (100 ng/ml) for 24h in the presence or absence of SU11274. OPN treatment of HepG3 cells significantly activated c-Met, Akt and ERK (Fig. 4F). Co-treatment with SU11274 resulted in significant inhibition of both basal and OPN-induced activation of c-Met and Akt (Fig. 4F). In the control group p-Akt/Akt ratio was 0.58 which was markedly downregulated to 0.05 upon SU11274 treatment. Treatment with OPN increased p-Akt/Akt ratio to 1.42 which was decreased to 0.63 upon SU11274 treatment. SU11274 treatment resulted in partial inhibition of basal and OPN-induced activation of ERK indicating that OPN might activate ERK independent of c-Met activation.

The findings with the chemical inhibitors were further confirmed by genetic means by establishing clones of LSF-17 cells stably expressing c-Met shRNA (LSF-17-shMet-1 and

LSF-17-shMet-17). Compared to the Control clone (LSF17-Consh-15) both total and activated c-Met levels were profoundly downregulated in LSF-17-shMet-1 and LSF-17-shMet-17 clones (Fig. 5A). Cell viability and Matrigel invasion was significantly inhibited in LSF-17-shMet-1 and LSF-17-shMet-17 clones compared to the Control clone (Fig. 5B and 5C, respectively). Similarly, transient transfection of c-Met siRNA to QGY-7703 cells also resulted in profound c-Met downregulation (Fig. 5D, inset), and inhibition of cell viability (Fig. 5D) and Matrigel invasion (Fig. 5E). These findings indicate that c-Met plays an important role in mediating *in vitro* phenotypes conferred by LSF.

Activation of Akt was significantly inhibited and activation of ERK was moderately suppressed in LSF-17-shMet-1 and LSF-17-shMet-17 clones compared to the Control clone (Fig. 5F). c-Met is a known inducer of EMT and therefore we analyzed the expression of EMT-associated genes upon c-Met knockdown. There was significant downregulation of Vimentin and upregulation of E-cadherin in the LSF-17-shMet-1 and LSF-17-shMet-17 clones compared to the Control clone indicating a reversal of EMT phenotype (Fig. 5F).

The *in vitro* findings demonstrating the importance of c-Met activation in mediating LSF function were further confirmed by *in vivo* assays. Compared to the Control clone, LSF-17-shMet-1 and LSF-17-shMet-17 clones formed distinctly smaller tumors when the cells were subcutaneously implanted in athymic nude mice (Fig. 6A). Tumors arising from LSF-17-shMet-17 clone showed areas of necrosis upon Hematoxylin & Eosin (H & E) staining (arrows, lowest panels, Fig. 6B). Immunohistochemical analysis of the tumors revealed marked downregulation of c-Met, the angiogenesis marker CD31 and the proliferation marker Ki-67 in sections of tumors arising from LSF-17-shMet-17 clone compared to the Control clone (Fig. 6B). LSF expression was unchanged in both clones indicating that c-Met inhibition could abrogate the oncogenic functions of LSF (Fig. 6B). A significant increase in apoptotic TUNEL-positive cells was detected in the tumors arising from the LSF-17-shMet-17 clone compared to the Control clone (Fig. 6C). However, the increase in apoptosis was not as profound as the inhibition of tumor growth indicating that inhibition of proliferation and angiogenesis might play a major role in growth inhibition of LSF-17-shMet-17 tumors. The metastatic ability of these clones was analyzed by the tail vein metastasis assay. When injected intravenously the Control clone formed multi-organ metastatic tumors, which were visible macroscopically from outside the animals (Fig. 6D, top panel, arrow). However, no metastatic tumors were formed by intravenous injection of the LSF17-shMet-17 cells. Analysis of lung sections indicated microscopic nodules throughout the lungs (Fig. 6D, bottom panel, arrow) in the Control clone injected animals, but very few lung nodules were detected in animals receiving the LSF17-shMet-17 clone.

Discussion

Although LSF was first cloned in 1987 as a cellular transcription factor no prior studies implicated LSF in carcinogenesis [15, 16]. Our finding that LSF functions as an oncogene for HCC and mediates its action by transcriptionally upregulating OPN was the first report connecting LSF to the carcinogenesis process [3]. In the present manuscript, we significantly extend those observations by documenting that LSF-induced secreted OPN profoundly activates c-Met, an important mediator of EMT, invasion and metastasis [6]. Our activated RTK array analysis identified robust activation of c-Met in LSF-overexpressing cells. LSF did not increase total c-Met level nor did it increase the level of its ligand HGF, thus ruling out two of the most common mechanisms of c-met activation. We focused on OPN as a potential activator of c-Met, since there was one report in the literature [17] demonstrating c-Met activation upon OPN treatment of human mammary epithelial cells. Interestingly, OPN has been shown to be transcriptionally induced by HGF in murine liver cells and to mediate HGF-induced invasive growth [18, 19]. We found that treatment of

HepG3 cells with OPN resulted in sustained and robust activation of c-Met. Since both c-Met and OPN can signal through CD44 receptors [4, 6] we reasoned that CD44 might be the common factor controlling OPN-induced c-Met activation. The extracellular domain of CD44 is required for c-Met autophosphorylation while the intracellular domain of CD44 recruits the ezrin, radixin and moesin (ERM) proteins to the HGF-c-Met-CD44 complex [11, 12]. The recruitment of ERM proteins leads to activation of guanine nucleotide exchange factor Sos that activates Ras and MEK/ERK. Binding of OPN to CD44 activates PLC γ , PKC and the PI3K-Akt pathway [4]. We propose a pathway in which OPN might replace HGF initiating interaction between CD44 and c-Met leading to Met autophosphorylation and activation. Indeed, our studies with neutralization antibodies documented that inhibition of CD44, and not the other OPN receptor $\alpha\text{v}\beta\text{3}$ integrin, prevented OPN-induced c-Met activation. We demonstrated that in the absence of OPN, CD44 and c-Met interact. However, in the presence of OPN the association of phospho-c-Met with CD44 increased. This observation was documented in HepG3 cells that express very low levels of OPN under serum-free conditions to remove potential contributions of OPN in the serum-containing medium as well as in LSF-17 and QGY-7703 cells. These findings indicate that binding of OPN to CD44 might lead to a conformational change in c-Met thereby resulting in autophosphorylation of c-Met in Y1234 and Y1235 residues in the catalytic domain (Fig. 6E). We observed that inhibition of c-Met in LSF17 cells resulted in greater suppression of Akt activation, compared to suppression of ERK activation, indicating that OPN-CD44-c-Met signaling predominantly activates PI3K/Akt signaling. What adapter proteins get recruited to c-Met to activate this important signaling cascade upon OPN binding to CD44 is an area we are currently exploring.

Inhibition of c-Met profoundly abrogated LSF functions, including *in vitro* proliferation and Matrigel invasion, and *in vivo* tumor growth, angiogenesis and metastasis, confirming the importance of c-Met activation in mediating LSF action. In clinical HCC samples, we observed a statistical correlation between LSF, OPN, and activated c-Met levels, further strengthening the significance of our findings. LSF is overexpressed in greater than 90% of human HCC samples compared to normal liver. As such, inhibition of either LSF or its downstream mediators, such as OPN or c-Met, might provide an effective strategy to counteract the pathogenesis of HCC. Indeed, a Phase I clinical trial using a specific c-Met inhibitor GSK089 for liver cancer [13] is now ongoing and our present findings strongly support the rationale for this potential therapeutic approach. However, a small molecule inhibitor that might bind to the DNA-binding domain of LSF and inhibit its transcriptional activity might also be an excellent way to block LSF, OPN and c-Met and might represent a potent HCC therapeutic. Studies are ongoing to identify such inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LSF	Late SV40 Factor
OPN	Osteopontin
RTK	Receptor tyrosine kinase

Fig. 1. LSF activates c-Met. **A.** Lysates from Control-8 and LSF-17 clones of HepG3 cells were used to detect activated receptor tyrosine kinases (RTKs) using a Proteome Profiler™ Array kit. **B.** Control-8 (Cont-8) is a neomycin-resistant clone while LSF-1 and LSF-17 clones are LSF-overexpressing clones of HepG3 cells. Control-7 (Cont-7) is a neomycin-resistant clone, while LSFdn-8 (dn-8) and LSFdn-15 (dn-15) clones are dominant negative LSF-overexpressing clones of QGY-7703 cells. Western blot analysis was performed to detect the expression of the indicated proteins in these cells. **C.** Expression of LSF and p-c-Met was determined by immunohistochemical analysis in FFPE sections of normal liver and matched HCC. A representative figure is shown from one patient. **D.** Statistical correlation between LSF and phospho-c-Met levels in 50 human HCC patients was analyzed by Pearson's test.

Fig. 2. OPN activates c-Met. A. HepG3 cells were treated with OPN (100 ng/ml) for the indicated time points and p-c-Met and total c-Met levels were analyzed by Western blot analysis. B. Expression of OPN and p-c-Met was determined by immunohistochemical analysis in FFPE sections of normal liver and matched HCC. A representative figure is shown from one patient. C. Statistical correlation between OPN and phospho-c-Met levels in 50 human HCC patients analyzed by Pearson's test. D. Left panel: LSF-17consh-15 (Consh-15) is a clone of LSF-17 cells that stably expresses control scrambled shRNA and LSF-17-OPNsh-6 (OPNsh-6) and LSF-17-OPNsh-18 (OPNsh-18) are clones of LSF-17 cells stably expressing OPN shRNA. The expression of the indicated proteins was detected by Western blot analysis. Right panel, LSF-17 clone of HepG3 cells were treated with normal IgG (mouse and rabbit; Santa Cruz), anti- $\alpha\beta$ 3 integrin antibody (mouse; Millipore) or anti-CD44 antibody (rabbit; Santa Cruz) at a concentration of 20 μ g/ml for 24 h and the expression of the indicated proteins was detected by Western blot analysis.

Fig. 3.

OPN facilitates c-Met activation via CD44 receptor. A. Left panel, HepG3 cells were cultured in serum-free conditions. Right panel, HepG3 cells were cultured in serum-containing medium and untreated or treated with OPN (100 ng/ml) for 24 h. Cell lysates were subjected to immunoprecipitation by anti-c-Met antibody and immunoblotting with anti-CD44, anti- α v β 3 integrin and anti-c-Met antibodies. B. The culture conditions are the same as in A. Immunoprecipitation was done with anti-CD44 antibody and immunoblotting was done with anti-c-Met and anti-CD44 antibody. C. HepG3 cell were cultured in serum-free conditions and then untreated or treated with OPN (100 ng/ml) for 24 h. Immunoprecipitation was done with anti-CD44 antibody and immunoblotting was done with anti-p-c-Met or anti-CD44 antibodies. D. Lysates from LSF-17 and QGY-7703 cells were subjected to immunoprecipitation by anti-c-Met antibody and immunoblotting by anti-CD44, anti- α v β 3 integrin and anti-c-Met antibodies. E. Lysates from LSF-17 and QGY-7703 cells were subjected to immunoprecipitation by anti-CD44 antibody and immunoblotting by anti-c-Met, anti-p-c-Met and anti-CD44 antibodies. The bottom panel serves as inputs for D and E. IgG was used as a control in all the experiments.

Fig. 4. Inhibition of CD44 and c-Met by SU11274 blocks LSF function. **A.** LSF-17 cells were treated with normal IgG or anti-CD44 antibody and then subjected to Matrigel invasion assay. **(B, C)** Cell viability (MTT) assay of LSF-17 (**B**) and QGY-7703 (**C**) cells treated with SU11274 (5 μM) for the indicated time period. **(D, E)** Matrigel invasion assays of LSF-17 (**D**) and QGY-7703 (**E**) cells treated with SU11274 (5 μM). For SU11274 removal, cells were treated with SU11274 for 24 h before seeding them in the invasion chamber in SU11274-free condition. For **A-E**, data represent mean ± SEM. **F.** HepG3 cells were untreated or treated with OPN (100 ng/ml) and with SU11274 (5 μM) for 24 h and the expression of the indicated proteins were detected by Western blot analysis. Asterisk (*) represents statistically significant difference ($p < 0.05$).

Fig. 5. Genetic inhibition of c-Met by shRNA blocks LSF function. LSF-17-Consh-15 (Control) is a clone of LSF-17 cells stably expressing control scrambled shRNA and LSF-17-shMet-1 (shMet-1) and LSF-17-shMet-17 (shMet-17) are clones of LSF-17 cells stably expressing c-Met shRNA. A. Expression of the indicated proteins was detected in the indicated cells by Western blot analysis. Cell viability (B) and Matrigel invasion (C) assays were performed with the indicated cells. D. QGY-7703 cells were transfected with control scrambled siRNA (siCon) or c-Met siRNA (siMet) and cell viability was measured by MTT assay. The inset shows Western blot analysis to detect effective knockdown of c-Met by the siRNA. E. Matrigel invasion assay of QGY-7703 cells transfected with control or c-Met siRNA. For B-E, data represent mean $\pm$ SEM. F. The expression of the indicated proteins was detected in the indicated cells by Western blot analysis. Asterisk (*) represents statistically significant difference ($p < 0.05$).

Fig. 6. Inhibition of c-Met blocks LSF-mediated tumorigenesis and metastasis *in vivo*. **A.** The indicated cells were subcutaneously implanted in athymic nude mice. Tumor volume and tumor weight were measured 3 weeks after implantation. The inset shows representative tumors at the end of the study. **B.** The expression of the indicated proteins was detected in tumor sections from Control and shMet-17 cells by immunohistochemical analysis. Arrows in the panel of CD31 staining denote microvessels. Arrows in the panel of H & E staining indicate areas of necrosis. **C.** Apoptotic (TUNEL positive) cells were detected in tumor sections induced by the indicated cell type. **D.** The indicated cells were intravenously injected into athymic nude mice and tumors were allowed to develop for 3 weeks. Inset: Top panel, macroscopic tumors in the animals (arrow). Bottom panel, sections of the lungs showing microscopic tumor nodule (arrow). Graphical representation of the number of metastatic nodules. For, A, C and D, data represent mean $\pm$ SEM. **E.** A schematic representation of c-Met activation by LSF and OPN. See text for details. Asterisk (*) represents statistically significant difference ($p < 0.05$).