Transcription factor Late SV40 Factor (LSF) functions as an oncogene in hepatocellular carcinoma

Byoung Kwon Yoo, Luni Emdad, Rachel Gredler, Christine Fuller, Catherine I. Dumur, Kimberly H. Jones, Colleen Jackson-Cook, Zao-zhong Su, Dong Chen, Utsav H. Saxena, Ulla Hansen, Paul B. Fisher, and Devanand Sarkar

Departments of Human and Molecular Genetics and Pathology, Virginia Commonwealth University Institute of Molecular Medicine, and Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, VA 23298; Department of Neurosurgery, Mount Sinai Medical Center, New York, NY 10029; and Department of Biology, Boston University, Boston, MA 02215

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Hepatocellular carcinoma (HCC) is one of the five most common cancers worldwide (1). The incidence of HCC is increasing despite a decrease in overall incidence of all cancers (2, 3). In the United States, the estimated new cases of HCC for 2008 were 21,370, of which 18,410 were expected to die (2). The mortality rate of HCC parallels that of its incidence because HCC is a tumor with rapid growth and early vascular invasion that is resistant to conventional chemotherapy, and no systemic therapy is available for the advanced disease (4). As such, understanding the molecular mechanism of HCC development and progression is imperative to establish novel, effective, and targeted therapies for this highly aggressive cancer. Our recent studies reveal that astrocyte elevated gene-1 (AEG-1) is overexpressed in >90% of human HCC patients, compared to normal liver, and AEG-1 plays a key role in regulating development and progression of HCC (5). The transcription factor Late SV40 Factor (LSF) was identified as a downstream gene of AEG-1, and we demonstrated that LSF mediates, in part, AEG-1-induced resistance to 5-fluorouracil (5-FU) in HCC cells (5, 6).

LSF, also known as LBP-1c and TFCP2, regulates diverse cellular and viral promoters (7, 8). A major cellular target of LSF is the thymidylate synthase (TS) gene, which encodes the rate-limiting enzyme in the production of dTTP, required for DNA synthesis (9). Inhibition of LSF abrogates TS induction and induces apoptosis. Thus, LSF plays an important role in DNA synthesis and cell survival. In the liver, LSF is activated by inflammatory cytokines and regulates the expression of acute phase proteins (10, 11). As yet, no studies have linked LSF to the process of tumorigenesis. However, several findings suggest a potential role of LSF in this process. LSF facilitates entry into G1/S phase of the cell cycle, promotes DNA synthesis, and functions as an antiapoptotic factor (9). Overexpression of LSF might augment all of these effects, thus promoting transformation and cancer cell survival. Additionally, because most HCCs are generated in the background of HBV or HCV infection, the activation of LSF by inflammatory cytokines that are secreted upon viral infection suggests that LSF might also play a role in the pathogenesis of inflammatory aspects of HCC. We therefore performed a detailed experimental analysis to elucidate the role of LSF in hepatocarcinogenesis.

Results and Discussion

Whereas in normal hepatocytes LSF protein expression was virtually undetected, its expression was robustly up-regulated in human HCC cells, except HepG3 cells, which do not form tumors in nude mice (Fig. 1A) (5). These findings were extended by tissue microarrays containing 86 primary HCC, 23 metastatic HCC, and 9 normal adjacent liver samples that were immunostained using anti-LSF antibody. Little to no LSF immunostaining was detected in the 9 normal liver samples, whereas significant LSF staining was observed in HCC samples (Fig. 1B). LSF expression was detected predominantly in the nucleus (Fig. S1). Among the 109 HCC samples, only 9 scored negative for LSF, and the remaining 100 (91.7%) showed variable levels of LSF expression. These findings suggest that as a downstream gene of AEG-1, LSF is a key regulatory factor in the pathogenesis of HCC.

Amplification of chromosome band 12q13, the location of the LSF gene, has been reported in some cases of HCC (13, 14). To examine the possibility that copy number gain might be the underlying mechanism of LSF protein overexpression in human HCC patients, dual-color fluorescence in situ hybridization (FISH) was performed on 5 HCC samples with high LSF expression. FISH analysis detected amplification of chromosome band 12q13 in all 5 samples, consistent with recent reports (13, 14).

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1To whom correspondence should be addressed. E-mail: dsarkar@vcu.edu.
expression in HepG3 cells, we established stable cell lines expressing LSF. Several of these clones were analyzed for LSF overexpression, among which LSF-1 and LSF-17 clones showed LSF expression that is comparable to a naturally LSF-overexpressing cell line, such as QGY-7703 (Fig. 2A). The nuclear expression of LSF was confirmed in LSF-17 clone by immunofluorescence (Fig. S2D). The luciferase activity of LSF WT-luc, a luciferase reporter construct containing four LSF-binding sites, was significantly higher in LSF-1 and LSF-17 clones compared to control neomycin-resistant clones Control-8 and Control-13 (Fig. 2B). Both LSF-1 and LSF-17 clones showed higher proliferative activity (Fig. 2C), anchorage-independent growth in soft agar (Fig. 2E), and Matrigel invasion abilities (Fig. 2F) compared to Control-8 and Control-13 clones. Interestingly, LSF overexpression resulted in chromosomal instability in HepG3 cells as evidenced by a significantly increased frequency of micronuclei in the LSF-1 clone (P < 0.05) (Fig. S3).

As complementation to the LSF-overexpressing clones, we established stable clones of QGY-7703 cells expressing a dominant-negative LSF (LSFdn, a double amino acid substitution mutant of LSF initially named 234QL/236KE that is unable to bind DNA) (9). An increased level of LSF expression over the control clones indicated expression of LSFdn. LSFdn-8 and LSFdn-15 clones expressed significantly higher levels of LSFdn compared to neomycin-resistant control clones Control-1 and Control-7 (Fig. 3A). The authenticity of these clones was confirmed by lack of activity of LSF WT-luc in LSFdn-8 and LSFdn-15 clones compared to Control-1 and Control-7 clones (Fig. 3B). Compared to Control-1 and Control-7 clones, LSFdn-8 and LSFdn-15 clones had slower proliferation rate (Fig. 3C), less

To assess the strength of association between LSF expression and stages of HCC, an ordinal logistic regression was conducted with the stage of HCC as the ordinal response and LSF expression as the independent variable in the proportional odds model. The hypothesis of association is highly significant: P < 0.001 using Pearson's χ² test with Yates’s continuity correction. A total of 109 HCC cases were analyzed.

Table 1. Immunoperoxidase staining of normal liver and different stages of HCC by tissue microarray using anti-LSF antibody: Intensity of LSF staining  

|                  | 0   | +   | ++  | +++ | Total cases |
|------------------|-----|-----|-----|-----|-------------|
| Normal liver     | 4   | 5   |     |     | 9           |
| Stage I HCC      | 4   | 15  | 3   | 1   | 23          |
| Stage II HCC     | 1   | 14  | 8   | 2   | 25          |
| Stage III HCC    | 3   | 15  | 15  | 5   | 38          |
| Stage IV HCC     | 1   | 4   | 8   | 10  | 23          |

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Overexpression of LSF increases and inhibition of LSF decreases tu-
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morigenesis of human HCC cells in nude mice. Control-8, LSF-1, and LSF-17 clones of HepG3 cells were s.c. implanted in athymic nude mice. Tumor volume (A) and tumor weight (B) were measured 3 weeks after implanta-
tion. Control-1, Control-7, LSFdn-8, and LSFdn-15 clones of QGY-7703 cells were s.c. implanted in athymic nude mice. Tumor volume (C) and tumor weight (D) were measured 3 weeks after implantation.

Immunofluorescence analysis of LSF, Ki-67, and CD31 in tumor sections of QGY-7703 clones of HepG3 cells (E) and Control-7 and LSFdn-15 clones of QGY-7703 cells (F).

In in vitro assays, the most significant effect of LSF over-
expression or inhibition was observed in the Matrigel invasion assay (Figs. 2 and 3). Because invasion is the first step in me-
tastasis, we evaluated the metastasizing capabilities of the established clones by the tail vein metastasis assay. Intravenous injection of LSF-1 and LSF-17 clones (figures shown only for LSF-17 clone) resulted in multiorgan macrometastasis, whereas no metastasis was observed for Control-8 clone of HepG3 cells (Fig. 5A). Metastasis was observed in the lungs (Fig. 5A Left), intestinal regions (Fig. 5A Center, arrow), and liver, and in the lower back region involving the vertebral column (Fig. 5A Right, arrow). The LSF-17-injected animals lost significant body weight (compare the size of the animals in Fig. 5A Center and Right), became cachexic, and started losing ~20% body weight (indication for euthanasia and considered as dead) 6 weeks after injection (Fig. 5B). As demonstrated by Kaplan–Meier survival curves, 80% of the animals injected with LSF-17 clone died by 12 weeks after injection, whereas none of the animals injected with the Control-8 clone of HepG3 cells died (Fig. 5B). Staining of the lungs showed preservation of normal alveolar architecture in Control-8-injected animals, whereas in LSF-17-injected animals the lungs were filled with a solid mass of infiltrating tumor cells adjacent to the blood vessels indicating that the tumor cells had extravasated, lodged into the lungs, and established colonies (Fig. 5D).

For QGY-7703 cells, the Control-1 and Control-7 clones gave rise to multiorgan metastatic tumors, whereas LSFdn-8 and LSFdn-15 clones did not show any external signs of metastasis (Fig. 5C). Staining of the lungs identified multiple solid nodules in Control-1-injected animals, whereas normal architecture was preserved in LSFdn-15-injected animals, with only a few isolated metastatic nodules (Fig. 5E).

To identify the downstream genes mediating the effects of LSF in HCC cells, gene expression profiles were compared be-
tween Control-8 and LSF-17 clones of HepG3 cells by Affyme-
triX microarray (GEO accession no. GSE19815). With a 20-fold cut-off, expression levels of 125 genes were up-regulated and those of 148 genes were down-regulated upon overexpression of LSF. Twenty-one of these genes are directly involved in the process of tumorigenesis (Fig. S4 and Table S1). The most robust induction was observed for SPP1, which encodes osteopontin (OPN), known to be important for regulating every step in me-
tastasis (15). The microarray data were confirmed by quantitative RT-PCR for several genes, showing an ~40-fold increase in OPN mRNA expression in the LSF-17 clone, as compared to the Control-8 clone (Fig. 6A). As a corollary, OPN mRNA expres-
sion was markedly down-regulated in the LSFdn-15 clone of...
Overexpression of LSF increases and inhibition of LSF decreases metastasis of human HCC cells in nude mice. (A) Control-8 and LSF-17 clones of HepG3 cells were injected i.v. through the tail vein in athymic nude mice. The internal organs were analyzed 4–6 weeks after injection. (B) Kaplan–Meier survival curve of animals injected with either Control-8 or LSF-17 clones of HepG3 cells. * mice losing ~20% body weight and euthanized (considered as dead). (C) Control-1 and LSFdn-15 clones of QGY-7703 cells were injected i.v. through the tail vein in athymic nude mice. Metastatic tumors were visible externally in mice injected with the Control-1 clone but not with the LSFdn-15 clone. (D) Graphical representation of metastatic lung nodules in the animals injected with Control-8 and LSF-17 clones of HepG3 cells. (Inset) H&E sections of lungs. (E) Graphical representation of metastatic lung nodules in the animals injected with Control-1 and LSFdn-15 clones of QGY-7703 cells. (Inset) H&E sections of lungs. For (D) and (E), the data represent mean ± SEM.

QGY-7703 cells compared to the Control-1 clone (Fig. 6B). Another LSF-downstream gene, complement factor H (CFH), also showed a similar trend (Fig. 6A and B). These findings were confirmed at the protein level by ELISA (Fig. 6C). The robust induction of OPN in LSF-overexpressing clones prompted us to hypothesize that LSF might regulate OPN expression at the transcriptional level. We scanned an ~1-kb region of the OPN promoter and identified two tandem LSF binding sites in this region (Fig. 6D). Consistent with that prediction, OPN promoter-luciferase reporter construct demonstrated significantly higher activity in the LSF-17 clone compared to the Control-8 clone (Fig. 6E) (16). Finally, a chromatin immunoprecipitation (ChIP) assay confirmed LSF binding to the OPN promoter (Fig. 6F).

To confirm the role of OPN in mediating LSF effect, we established stable OPN shRNA-expressing clones in the background of LSF-17 clone of HepG3 cells (LSF17-OPNsh). Two independent clones LSF17-OPNsh-6 and LSF17-OPNsh-18 showed marked down-regulation of OPN mRNA and protein expression (Fig. 7A and B, respectively) when compared to the parental LSF-17 clone or LSF-17consh-15 clone that stably expresses control scrambled shRNA. LSF expression remained unchanged in LSF-17, LSF-17consh-15, and LSF-17-OPNsh clones (Fig. S5). Compared to parental LSF-17 and LSF-17consh-15 clone, LSF17-OPNsh-6 and LSF17-OPNsh-18 clones had a significantly slower proliferation rate (Fig. 7C), less colony formation (Fig. 7D), anchorage-independent growth in soft agar (Fig. 7E), and Matrigel invasion abilities (Fig. 7F). LSF17-OPNsh-6 and LSF17-OPNsh-18 clones formed significantly smaller s.c. tumors in nude mice compared to the parental LSF-17 and LSF-17consh-15 clone (Fig. 8A). These studies were further corroborated by experimental metastasis assays demonstrating significantly decreased numbers of metastatic nodules in the lungs in mice injected with LSF17-OPNsh-18 clone compared with those injected with LSF-17consh-15 clone (Fig. 8B).

Because OPN is a secreted protein, we checked whether conditioned media from LSF-17 clones might augment the invasive ability of the parental HepG3 cells. Indeed, conditioned media from the LSF-17 clone, but not from the Control-8 clone, significantly increased invasion by HepG3 cells (Fig. 8C). OPN works through its canonical receptors (15). We blocked these receptors on LSF-17 clone of HepG3 cells with neutralizing antibodies and performed Matrigel invasion assay. Whereas normal IgG did not affect the invasive ability of the LSF-17 cells, anti-αvβ3 integrin or anti-CD44 receptors (15). We blocked these receptors on LSF-17 clone of HepG3 cells with neutralizing antibodies and performed Matrigel invasion assay. Whereas normal IgG did not affect the invasive ability of the LSF-17 cells, anti-αvβ3 integrin or anti-CD44 antibodies significantly inhibited invasion and the combination of the two antibodies decreased the invasion, further confirming that OPN working through its canonical receptors plays a key role in regulating LSF function (Fig. 8D). It should be noted that for all of the assays described in this article using isolated clones, similar in vitro phenotypes, although less pronounced because of transfection efficiency, were observed with transient transfection assays without selection, thereby ruling out any clonal bias arising from the selection procedure.

Our present findings reveal a role of LSF in the process of hepatocarcinogenesis. We demonstrate that by augmenting transcription of OPN, LSF promotes aggressive progression of HCC. OPN levels can be used as a sensitive and specific marker in predicting disease progression in diverse cancers, including HCC, and OPN is known to promote every step in metastasis as
well as growth of the primary tumor (15, 17). By regulating OPN expression, LSF functions as a key regulator of HCC development and progression. In addition, LSF also activates two important cell survival-regulating pathways, MEK/ERK and NF-κB (Fig. S6), and inhibition of the MEK/ERK pathway significantly abrogates invasion by LSF-17 cells (Fig. S7). Activation of NF-κB by LSF suggests its potential role in regulating the inflammatory aspects of HCC (18). Our present findings thus strongly suggest that LSF might be a viable target, and that small-molecule inhibitors targeting the DNA binding domains of LSF might be an effective HCC therapeutic. Additionally, the correlation of LSF expression with the stages and grades of HCC suggests that LSF might be used as a prognostic marker for this disease. Finally, the observation that LSF is overexpressed in cancer indications other than HCC indicates a potential oncogenic function of LSF in diverse other cancers (Fig. S8).

Materials and Methods
Cell Lines, Culture Condition, Viability, Colony Formation Assays, Anchorage-Independent Growth in Soft Agar, and Matrigel Invasion Assays. Primary rat hepatocytes were isolated and cultured as described in ref. 19. SNU-423 cells were obtained from ATCC and cultured as described. HepG3, QGY-7703, Hep3B, HuH7, Focus, and HEK-293 cells were cultured as described in ref. 5 and 20. Cell viability was determined by standard MTT assay as described in ref. 5. Colony formation, anchorage-independent growth in soft agar, and Matrigel invasion assays were performed exactly as described in ref. 5.

Tissue Microarray. Human HCC tissue microarrays were obtained from Imageon. Two tissue microarrays were used: one containing 40 primary HCC, 10 metastatic HCC, and 9 normal adjacent liver samples (IMH-360; Imageon), the other containing 46 primary HCC and 13 metastatic HCC (IMH-318; Imageon) for immunohistochemistry. IMH-360 was used for fluorescence in situ hybridization analysis (FISH).
Immunostaining. Immunofluorescence analysis in tumor sections was performed essentially as described in ref. 5. Anti-LSF (1:200, mouse monoclonal; BD Biosciences), anti-Ki-67 (1:200, mouse monoclonal; BD Biosciences), and anti-CDC2 (1:200, mouse monoclonal; Dako) antibodies were used. Images were analyzed using an Olympus immunofluorescence microscope. For the tissue microarray (IMH-360 and IMH-318; Imgenex), anti-LSF antibody was used at 1:100 dilution and the signals were developed by avidin-biotin-peroxidase complexes with a DAB substrate solution (Vector Laboratories).

Nude Mouse Xenograft Studies. Subcutaneous xenografts were established in the flanks of athymic nude mice using 1 × 106 human HCC cells and the clones. Tumor volume was measured twice weekly with a caliper and calculated using the formula π/6 × larger diameter × smaller diameter (2). Mice were followed for 3 weeks. For the metastasis assays, 1 × 106 cells were i.v. injected through the tail vein in nude mice. The lungs, intestines, liver, bone, and other organs were isolated and analyzed after 4 weeks. All experiments were performed with at least five mice in each group and repeated three times.

Total RNA Extraction, Real-Time PCR, and Microarray Assays. Total RNA was extracted using Qiagen miRNeasy mini kit. Real-time PCR was performed using a ABI 7900 fast real-time PCR system and Taqman gene expression assays for OPN, CHF, and GAPDH according to the manufacturer’s protocol (Applied Biosystems). An Affymetrix oligonucleotide microarray (GeneChip Human Genome U133A 2.0) analysis was performed to compare gene expression between Control-8 and LSF-17 clones of HepG3 cells using standard Affymetrix protocol (22).

Fluorescence in Situ Hybridization and Micronuclei Analysis. Dual-color fluorescence in situ hybridization (FISH) was performed as previously described on hepatocellular carcinoma tissue microarrays (23). Bacterial artificial chromosome (BAC)-derived test probes targeting LSF (12q13, RP11-142E3; BAC-PAC Resources Center) were paired with an enumeration probe for the pericentromeric region of chromosome 12 (D12Z3) for dual-target hybridization.

ChIP Assays. ChIP assays were performed using a commercially available kit from Active Motif according to the manufacturer’s protocol. OPN promoter-specific primers used were sense 5′-ACACGCTTATGGCGGATTG-3′ and antisense 5′-GAACTTGGTAGGGAAA-3′.

Statistical Analysis. Data were represented as the mean ± SEM and analyzed for statistical significance using one-way ANOVA followed by Newman-Keuls test as a post hoc test. To assess the strength of association between LSF expression and stages of HCC, an ordinal logistic regression was conducted with the stage of HCC as the ordinal response and LSF expression as the independent variable in the proportional odds model using Pearson’s χ2 test with Yates’s continuity correction.

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