Complement proteins C7 and CFH control the stemness of liver cancer cells via LSF-1

Hyang Sook Seol[a], **Sang Eun Lee**[a], **Joon Seon Song**[b], **Je-Keun Rhee**[c], **Shree Ram Singh**[d], **Suhwan Chang**[c], and **Se Jin Jang**[a,b]

[a]Asan Institute for Life Science, University of Ulsan College of Medicine, Asan Medical Center, Seoul, South Korea

[b]Department of Pathology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, South Korea

[c]Department of Biomedical Sciences, University of Ulsan College of Medicine, Asan Medical Center, Seoul, South Korea

[d]Basic Research Laboratory, Stem cell regulation and Animal Aging Section, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

Abstract

Tumor-initiating cells are important for the formation and maintenance of tumor bulks in various tumors. To identify surface markers of liver tumor-initiating cells, we performed primary tumorsphere culture and analyzed the expression of cluster of differentiation (CD) antigen genes using NanoString. Interestingly, we found significant upregulation of the complement proteins ($p=1.60 \times 10^{-18}$), including C7 and CFH. Further studies revealed that C7 and CFH are required to maintain stemness in liver cancer cells. Knockdown of C7 and CFH expression abrogated tumorsphere formation and induced differentiation, whereas overexpression stimulated stemness factor expression as well as *in vivo* cell growth. Mechanistically, by studying C7 and CFH-dependent LSF-1 expression and its direct role on stemness factor transcription, we found that LSF-1 is involved in this regulation. Taken together, our data demonstrate the unprecedented role of complement proteins on the maintenance of stemness in liver tumor-initiating cells.

Keywords

Correspondence to: Suhwan Chang; Se Jin Jang.

Contact Info:
Suhwan Chang, Ph. D., Tel: +82-2-3010-2095; Fax: +82-2-3010-8165, suhwan.chang@amc.seoul.kr
Se Jin Jang, MD, Ph. D., Tel: +82-2-3010-5966; Fax: +82-2-472-7898, jangsejin@amc.seoul.kr
Shree Ram Singh, Ph. D., Tel: +1-301-846-7331; Fax: +1-301-846-7017, singhshr@mail.nih.gov

Conflict of interests

The authors declare no competing financial interests.

1. Introduction

Liver cancer is the fifth most prevalent cancer and the second highest cause of cancer deaths in men worldwide [1]. Hepatocellular carcinoma (HCC) is the major histological subtype, accounting for 70–85% of cases of primary liver cancer. Although locoregional or surgical treatments and, in advanced cases, chemotherapy are used in clinical settings, the 5-year survival rate of HCC patients remains poor largely due to metastasis, recurrence, and resistance to chemo- and radiotherapy [2].

The high rate of recurrence and heterogeneity are the two major features of HCC [3]. Recent studies suggest that heterogeneity results from the hierarchical organization of tumor cells by a subset of cells with stem/progenitor cell features, which are known as cancer stem cells (CSCs) [4]. These CSCs within the bulk of the tumor demonstrate the capacity to self-renew, differentiate, and give rise to new tumors [5–7]. This also accounts for the hierarchical organization of heterogeneous cancer cells and a high rate of cancerous recurrence. However, how liver CSCs sustain self-renewal remains largely unknown. The process of HCC development by itself is a hard-to-describe phenomenon. However, accumulating evidence suggests that the surrounding environment is a very important factor in tumor incidence. Related to this, a crosstalk between inflammation and cancer is known to increase the risk of developing cancer [8, 9]. Specifically, liver CSCs are reportedly located in specialized microenvironments within tumors where inflammatory cells and factors are essential components [10–13].

The complement system is one of the basic components of the innate immune system, in which many proteins work as a cascade to form a complex pore structure. The complement proteins in the plasma are mainly synthesized in the hepatocytes, but they are also secreted by endothelial cells, white blood cells, and epithelial cells [14–17]. In the extravascular tissues, the complement proteins also participate in cell-to-cell communications and are involved in organ regeneration, angiogenesis, epithelial-mesenchymal transition, and cell migration. Markiewski et al [18] demonstrated that regulatory T cells (Tregs) are activated in breast tumors by the C5a receptor protein in the tumor microenvironment, which is a component of the classical complement cascade [18]. Further, a peptide antagonist of the C5a receptor enhances CD8+ T-cell antitumor responses and is as effective as the chemotherapeutic paclitaxel (Taxol) in retarding tumor growth [19]. Here, we also report a novel role for complement proteins; mainly C7 (complement component 7) and complement factor H (CFH). Using our PCR array results, we show that the C7 and CFH complements are upregulated in liver tumor-initiating cells (TICs). In addition, we show that these proteins are needed to sustain stemness in liver TICs and control stemness factors via late SV40 factor (LSF-1).

2. Materials and methods

2.1. DNA constructs and antibodies

The primary antibodies and dilutions included the following: anti-Nanog (1:1000; sc-134218, Santa Cruz Biotechnology), anti-Oct4A (1:1000; #2840, Cell Signaling), anti-Sox2 (1:1000; sc-20088, Santa Cruz Biotechnology), anti-c-Myc (1:1000; #9402, Cell

Signaling), anti-C7 (1:1000; GTX63807, Genetex), anti-CFH (1:500; GTX63521, Genetex), and anti-LSF-1 (1:1000; 610818, BD Transduction Laboratories). For the immunohistochemistry analysis, anti-CK7 (1:400; M7018, DAKO), anti-p-CEA (1:600; A0115, DAKO), and anti-Hep Par1 (1:200; M7158, DAKO) were used. Staining was performed using Alexa-488 or Alexa-555 (Molecular Probes), and the immunofluorescence-stained cells were observed using fluorescence microscopy (Zeiss LSM 710). The expression vectors containing the human C7, CFH, and LSF-1 sequences were purchased from GE Healthcare (C7: MHS6278-202760004, clone ID: 6184213; CFH: MHS6278-202800294, clone ID: 40148771; LSF-1: OHS5898-219582217, clone ID: PLOHS_100073456; all from Dharmacon).

2.2. Primary cancer cell culture-derived patient tissues

Primary cancer cells were originated from surgically resected from primary liver cancer tissues collected by Asan Bio Resource Center under ethical approval by the institutional review board of Asan Medical Center (IRB-20120112). Before freezing of the liver specimens for banking, a small piece of tumor tissue was separated and processed to establish primary cancer cell lines. Briefly, the tumor tissues were minced with scissors and subsequently digested using 1 mg/mL of type IV collagenase (Sigma Chemical Co., St. Louis, MO) in DMEM/F12 for 60 minutes at 37°C. After incubation, the tissues were washed with medium containing 10% fetal bovine serum (16000-044; Life Technologies). To promote the adhesion and growth of the epithelial tumor cells, Hepatocyte Basal Medium (HBM; Lonza, Walkersville, MD) containing human epidermal growth factor (hEGF), hydrocortisone, insulin, transferrin, GA-1000, ascorbic acid, BSA-FAF, and 10% FBS was used to culture the primary liver cancer cells, which were plated on to a collagen type 1 dish in a humidified incubator at 37°C under a 5% CO_2 atmosphere. For sphere culture in a low-attachment dish, we used HBM (−FBS) with 1XN2, 1XB27, and 10uM Rock inhibitor and 5nM HGF as the culture media.

2.3. Cell line culture

Four liver cancer cell lines (SNU398, SNU423, SNU449, and Huh7) were grown in RPMI 1640 medium, and two liver cancer cell lines (Hep3B and HepG2) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.4. Mouse xenograft tumor model analysis

All four sphere-cultured cells were implanted in 6–8-week-old NOD/SCID mice (Charles River Laboratories, Wilmington, MA). Briefly, 3×10^3 patient-derived sphere cancer cells were suspended in 100ul Matrigel (BD Biosciences, San Jose, CA) and injected into the subcutaneous layer on the backs of NOD/SCID mice. After 3–4 months, when the tumor size reached >1cm³, the mice were anesthetized via an intraperitoneal injection of a mixture of 40mg/kg Zoletil (Virbac Laboratories, BP 27-06511 Carros, France) and 5mg/kg Rum pun (Bayer Korea, South Korea), and the tumors were surgically removed.

2.5. Morphology and immunohistochemistry assessment

Morphological comparisons between the original and engrafted tumors obtained from the sphere cells were performed by two independent pathologists. On H&E staining, the identification of pathologic type, differentiation grading, and tumor architecture were evaluated. For IHC comparisons between the original and xenograft tumors, immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections was performed using an automatic immunohistochemical staining device (Benchmark XT; Ventana Medical Systems, Tucson, AZ). Briefly, 4-μm-thick 2D cultured tissue sections were transferred onto poly-L-lysine-coated adhesive slides and dried at 74°C for 30 minutes. After epitope retrieval by heating for 1 hour in ethylene diamine tetra-acetic acid (pH 8.0) in the autostainer, the samples were incubated with the indicated antibodies. The sections were subsequently incubated with secondary antibodies, and then visualized using an ultra View Universal DAB Detection kit (Ventana Medical Systems, Inc). The nuclei were counterstained with Harris hematoxylin.

2.6. Flow cytometry

The primary and sphere-cultured cells were resuspended in PBS and incubated with 0.1% BSA blocking reagent for 30 minutes. Then, the cells were stained with directly conjugated monoclonal antibodies, anti-human CD133-PE, anti-human EpCAM-PE, anti-human-CD90-APC (Miltenyi Biotec, Germany), anti-human CD44-FITC, and anti-human CD49f-FITC (BD Biosciences) for 60 minutes at 4°C. The control was incubated in parallel. Flow cytometry analysis was performed on BD FACS Canto™ (BD Biosciences) at the Asan Life Science Lab and Flow cytometry Core Facility of Asan Medical Center.

2.7. NanoString analysis

Total RNA was extracted from patients' primary cultured cells and sphere-cultured cells at 7 days. cDNA microarray analysis was conducted using then Counter GX Human Immunology Analysis system (NanoString Technologies, Inc).

2.8. RNA extraction and qRT-PCR

Total RNA was isolated from the cells and tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). For the single-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using MultiScribe™ Reverse Transcriptase (Applied Biosystems, Carlsbad, CA). The primer sets and amplification conditions for PCR are listed in Supplementary Table S1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA and 18s ribosomal RNA expression levels were used as endogenous controls. The expression levels of the genes were normalized against those of the endogenous control using the $2-\Delta C_t$ method, and $p < 0.05$ is considered statistically significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.9. Immunoblot analysis

Cells were lysed using the Cell Lysis Buffer (#9803; Cell Signaling Technology, Danvers, MA) with a protease inhibitor cocktail kit (P3100-005; GenDEPOT, Barker, TX) and a phosphatase inhibitor (sc-45065; Santa Cruz Biotechnology, Santa Cruz, CA). Aliquots containing 20 μg of cell lysates were electrophoretically resolved on SDS-polyacrylamide

gels and then transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK) in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v]methanol [pH 8.3]) at 60V and 4°C for 180 minutes. The membranes were blocked with 5% BSA in PBS containing 0.1% Tween-20 for 60 minutes at room temperature, and then incubated with the indicated antibodies. The secondary antibody included HRP-conjugated goat anti-rabbit IgG (1:5000; ADI-SAB-300-J, Enzo Life Bioscience) or goat anti-Mouse IgG (1:5000; ADI-SAB-100-J, Enzo Life Bioscience). The blots were developed using the ECL western blotting analysis system (GE Healthcare, Buckinghamshire, UK).

2.10. Synthetic siRNA transfection

Two liver cancer cell lines, SNU449 and Huh7, were transfected with siRNAs targeting C7, CFH, LSF-1, or a scrambled sequence siRNA to a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were harvested for assay at 7 days after transfection. The human C7, CFH, and LSF-1 siRNA sequences included the following:

C7 #1

forward primer: 5′-ACAUAGAACUUACUGGAAAUU-3′

reverse primer: 5′-UUUCCAGUAAGUUCUAUGUUU-3′)

C7 #2

forward primer: 5′-CAUAGAACUUACUGGAAAUUU-3′

reverse primer:5′-AUUUCCAGUAAGUUCUAUGUU-3′)

CFH #1

forward primer: 5′-GCAAAGAAGUGAAAGUGGAUU-3′

reverse primer: 5′-UCCACUUUCACUUCUUUGCUU-3′)

CFH #2

forward primer: 5′-ACACAGAACUGGAGAUGAAUU-3′

reverse primer: 5′-UUCAUCUCCAGUUCUGUGUUU-3′)

LSF-1 #1

forward primer: 5′-GCAGAUUUAUUGAAAUUAAUU-3′

reverse primer: 5′-UUAAUUUCAAUAAAUCUGCUU-3′)

LSF-1 #2

forward primer: 5′-GUAGAAACUCUACAUAAUUUU-3′

reverse primer: 5′-AAUUAUGUAGAGUUUCUACUUU-3′

LSF-1 #3

forward primer: 5′-GGAAUUGUGUGAUGUUUAAUU-3′

reverse primer: 5′-UUAAACAUCACACAAUUCCUU-3′

Scrambled siRNA

forward primer: 5′-CCUCGUGCCGUUCCAUCAGGUAGUU-3′

reverse primer: 5-CUACCUGAUGGAACGGCACGAGGUU-3′

2.11. Generation of stable cell lines

The control plasmid and the C7, CFH, and LSF-EGFP-C1 expression vectors were transfected into SNU449 and Huh7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The cells were selected by incubation with G418 (Invitrogen; 800 μg/mL for SNU449 and 500 μg/mL for Huh7) for > 4 weeks in order to obtain drug-resistant clones. Stable single clones were picked, and the C7, CFH, and LSF-1 expression levels were assessed using western blot analysis.

2.12. Chromatin immunoprecipitation

SNU449 and Huh7 cells were cross-linked with 1% formaldehyde and then incubated in lysis buffer (50mM Tris-HCl [pH 8.1], 1% SDS, 10mM EDTA, and protease inhibitor cocktail) on ice for 10minutes. After sonication (Sonicsvibra-cell, VCS X 130), the samples were immunoprecipitated with anti-LSF1 antibody or normal anti-mouse IgG (N103; Oncogene). The DNA was eluted and purified using a PCR purification kit, and PCR was performed using specific primers in order to amplify the LSF1-binding sites of the stemness promoters (Oct-4, 5′-ATT CTG TGT GAG GGG ATT GG-3′, 5′-GAC ATC TAA TAC CAC GGT AGG-3′; SOX2 5′-GGA TAA CAT TGT ACT GGG AAG GGA CA-3′, 5′-CAA AGT TTC TTT TAT TCG TAT GTG TGA GCA-3′ and c-Myc 5′-GCC TGC GAT GAT TTA TAC TCA C-3′, 5′-AAA CAG AGT AAG AGA GCC G-3′) primers.

2.13. Immunofluorescence microscopy

Cells were washed twice with PBS and fixed with 4% formaldehyde (Sigma F8775-25ML) for 30 minutes at room temperature, washed again three times with PBS, and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 minutes. The cells were washed three times with PBS and blocked with 3% BSA in PBS for 1 hour. Thereafter, the cells were incubated with a primary antibody and a secondary antibody for 1 hour each, with three washes in between incubations. Nuclei were counterstained with PI. Images were acquired using ZEN 2012 software and x40 oil immersion objective lens.

2.14. Transient transfection and luciferase assay

Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For the luciferase reporter assays, the cells were plated onto 24-well plates and transfected with an empty vector (pGL3-basic) the next day, pGL3-C7 promoter (luciferase reporter plasmid containing C7 promoter), pGL3-CFH promoter, pGL3-LSF-1 promoter, and Renilla luciferase expression plasmid (SV40-Luc) as the internal control. The luciferase assays were measured using a Dual Luciferase Reporter assay kit (E1910, Promega), according to the manufacturer's protocol.

2.15. Statistical analysis

Results are expressed as mean ± standard deviations (SD). The significance of differences between means was determined using a two-tailed Student's *t*-test. A *p*-value < 0.05 was considered statistically significant. Associations between categorical variables were analyzed by Fisher's exact tests. *P*-values less than 0.05 were considered statistically significant. SPSS 18.0 (SPSS, Chicago, IL) was used for statistical analyses.

3. Results

3.1. Enrichment of TICs from liver cancer specimens using tumorsphere culture

To identify novel surface markers in liver TICs, we cultured the primary tumor cells obtained from 4 patients (3 hepatocellular carcinomas and 1 combined hepatocellular-cholangiocarcinoma) on collagen coated plates for 2D culture and low-attachment plates supplemented with stem cell media for tumor sphere culture (see details in *Materials and Methods*). The attached cancer cells showed typical epithelial cell morphology, in contrast, the tumorspheres formed on low-attachment plates using stem cell media (as shown in Figure 1A). To determine if the tumor sphere culture was enriched by the TICs, we transplanted 3000 tumorsphere cells into NOD-SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mice. We observed tumors at 3–4 months after injecting the tumorsphere cells (Supplementary Figure S1A), thereby suggesting that the tumorsphere culture contains enriched TICs. Indeed, when we injected 3000 tumorspheres (S) and 2D cultured cells into NOD-SCID mice, we found tumors in 7 out of 8 tumorsphere cell xenografts, in comparison with only 2 out of 8 control (2D) cells (Supplementary Figure S1B). Histological analysis was next performed to examine if these xenograft tumors retain the characteristics of the original patients' tumor. The xenograft and primary tumors were examined using hematoxylin and eosin (H&E)-staining or immunostaining with antibodies against cytokeratin 7 (CK7), polyclonal carcinoembryonic antigen (p-CEA), and hepatocyte paraffin-1 (Hep Par1) to assess histological similarity (Figure 1B). H&E staining revealed similarities in basic cellular and histologic features between pairs. The IHC staining results for CK7, p-CEA, and Hep Par1 also support the idea that the xenograft tumor retains histological similarities with their parental tumors. Enrichment of the TICs in the tumorsphere was further supported by the real-time PCR results of known stemness factors, including Nanog, Sox2, and Oct4. As shown in Figure 2A–C, the tumorsphere cells expressed more stemness factors than the control cells, indicating that the CSC population is enriched. To confirm that these results are valid in liver cancer cells, we generated tumorsphere cultures from 6 liver cancer cell lines and confirmed the elevated mRNA level of the stemness factors (Figure 2D–F). These data indicate that the liver primary- or cell line-derived TICs are enriched by tumorsphere culture.

3.2. Complement, one of the critical elements of innate immunity, is upregulated in liver TICs

After confirming that the tumorsphere cultures are enriched with TICs, we examined the expression levels of known stem cell markers in the tumorsphere culture in comparison with 2D cultured cells using FACS (Fluorescence-activated cell sorting) analysis. Interestingly, we observed increased EpCAM (Epithelial cellular adhesion molecule) in only 2 of 4

tumorsphere cultures. CD49f and CD133 were also increased in only 1 of 4 tumorsphere cultures, respectively (Supplementary Table S2). Furthermore, CD90, a known liver cancer stem cell marker, was found to be unaltered in all tumorsphere cultures. Based on these results, we decided to screen for novel TIC markers in our liver tumorsphere cell population using NanoString Counter GX human immunology analysis. Unexpectedly, the NanoString results revealed that the complement and coagulation cascade genes were most significantly upregulated in the tumorsphere cells (Table 1, Supplementary Table S3 for raw data). Using real-time PCR analysis, we selected C7 and CFH, which were the most highly expressed complement proteins, in 2 of 3 primary tumorsphere cells (Figure 3A–B). Of note, the levels of the other complements, such as C3, C1S, and C1R, were also significantly elevated (Supplementary Figure S2A–C). We confirmed upregulated complement expression in the tumorsphere cultures of the liver cancer cell lines as well (Figure 3C–D, Supplementary Figures S3A–C). In addition to mRNA expression, we observed the increase in C7 and CFH protein expression in the tumorsphere cells (Figure 3E). Moreover, using confocal microscopy, we found that C7 and CFH were mainly located in the nucleus in tumorsphere cells (Figure 3F). These data demonstrate that C7 and CFH are complement factors that are enriched in the tumorsphere cells and suggest a novel function for complement proteins in the nucleus.

3.3. Complement C7 and CFH control stemness factor expression and tumorsphere formation

To determine the roles of C7 and CFH in liver TIC, we silenced these two complement genes using siRNA (Figure 4A). Notably, C7 and CFH depletion abrogated the formation of tumorspheres in SNU449 cells. On the other hand, C7 and CFH knockdown in Huh7 cells resulted in attached (i.e., differentiated) cells with fibroblast-like morphology (Figure 4A). In a line with these results, we observed that the levels of the stemness factors, including Nanog, Oct4, Sox2, and c-Myc increased with the transient expression of C7 or CFH (Figures 4B–C). Similarly, the stable expression of C7 and CFH also elevated the expression of the stemness factors, although there are cell line-specific differences (Figure 4D–E; see *Discussion*). Particularly, in case of C7 stable cells, we found Oct4 and c-Myc were upregulated in SNU449 cells. In the Huh7 cells, stable C7 expression resulted in Oct4, Sox2, and c-Myc upregulation (Figure 4D). Regarding stable CFH expression, we found Oct4, Nanog, Sox2, and c-Myc upregulation in SNU449 cells, whereas Oct4 and c-Myc were upregulated in Huh7 cells (Figure 4E). We also tested if the stemness factors control complement proteins, however, we did not find such regulation (Supplementary Figure S4A–D). Moreover, the C7 and CFH stable cell lines produced more tumorspheres in Huh7 cells than control cells (Figure 4F, where the black circle indicates the formed tumorsphere; quantification is shown in Figure 4G). These data indicate that C7 and CFH can upregulate stemness factors in order to sustain the stemness of liver cancer cells.

3.4. Identification of LSF-1 as a mediator of stemness factor control via complement proteins

In order to understand the role of complement in liver TICs, we focused on the underlying mechanism by which C7 and CFH control stemness factors. Because there is no known transcriptional activity of C7 and CFH, we hypothesized the presence of a mediating

transcription factor responsible for this regulation. Among the possible candidates, LSF-1 drew our attention because a recent study reported the oncogenic function of LSF-1 in liver cancer [20, 21]. Moreover, one of the known targets of LSF-1 is CFH [20], thereby suggesting a functional link. Therefore, we first tested the expression of LSF-1 in tumorsphere cells. As shown in Figures 5A–B, LSF-1 mRNA and protein expression were increased in both SNU449 and Huh7 tumorspheres. Interestingly, the results in Figure 5C showed the increased nuclear expression of LSF-1 in the tumorsphere cells. Using confocal analysis, we also observed that LSF-1 was mostly located in the nucleus, and its level is increased in tumorspheres (Figure 5D). Importantly, we observed the overexpression of C7 and CFH and upregulated LSF protein levels (Figure 5E–F), suggesting that LSF-1 is downstream of C7 and CFH. Indeed, when we knockdown C7 or CFH, we observed reduced LSF-1 along with the downregulation of the stemness factors (Figures 5G–H).

Next, we examined if LSF-1 affects the stemness of cancer cells by silencing it. LSF-1 knockdown abrogated tumorsphere formation in SNU449 and Huh7 cells, similar to that of the complement genes (Figure 5I). Western blot analysis showed that LSF-1 knockdown remarkably reduced the expression of the stemness factors Nanog, Oct4, Sox2, and c-Myc in both cells (Figure 5J). These data show that LSF-1 is a transcription factor that is induced by C7 or CFH and plays a role in the regulation of stemness factor expression in liver TICs.

3.5. LSF-1 directly controls the Nanog, Oct4, Sox2, and c-Myc promoters, and its expression augment tumorsphere formation and in vivo tumor growth

To further understand the role of LSF-1in stemness factor expression, we obtained the promoter reporter plasmids for Nanog, Oct4, Sox2, and c-Myc (obtained from Dr. Janghwan Kim [KRIBB, Taejeon, South Korea]) and tested if LSF-1 activates these promoters. The results presented in Figures 6A–B show that LSF-1 does indeed transactivates the Nanog, Oct4, Sox2, and c-Myc promoters in both SNU449 and Huh7 cells. Furthermore, using the ChIP assay, we found that LSF-1 interacts with the promoters of the Oct4, Sox2, and c-Myc genes (Figure 6C). These data suggest that LSF-1 directly controls the transcription of stemness factors. Furthermore, in stable LSF-1 cell lines, we found the increased expression of stemness factors (Figure 6D) and enhanced tumorsphere formation (Figure 6E; see Figure 6F for quantification). Lastly, when the stable LSF-1 cell line was xenografted onto mice, we observed a significant increase in tumor size in comparison with the control cells (Figure 6G; see Figure 6H for quantification). Altogether, these data indicate LSF-1 acts as an oncogene by increasing the stemness of liver cancer cells.

3.6. C7, CFH, and LSF-1 are highly expressed in HCC tissues

To confirm our findings in clinical samples, we analyzed the expression of C7, CFH and LSF-1 in 20 HCC/corresponding non-tumor tissue pairs retrieved from the Bio-Resource Center at Asan Medical Center. The non-tumorous liver parenchyma analyzed for this study demonstrated cirrhosis (n=11), chronic hepatitis (n=3), or minimal histologic change (n=6), respectively. The data were anonymous, and the clinicopathologic characteristics of 20 patients were shown in Table 2. Since normal liver is a major source of complement proteins, we focused on expression of the proteins in tumor tissues in comparison with surrounding non-neoplastic liver tissues. We found that 16 (80%) tumor samples highly

expressed LSF-1 over the levels of corresponding normal tissues (Figure 7A and Supplementary Figure 6C for quantitation)) and associated with high expression of CFH and/or C7 (Supplementary Figure 6A and B for quantitation). As seen in xenograft experiment, LSF-1-high tumor showed larger tumor size (7.3 cm vs 4.3 cm). As LSF-1 expression is functionally linked to C7 and CFH in our cell line experiment, we allocated 20 patients into two groups: C7/CFH/LSF-1-high group (n=8) and low group (n=12), and analyzed clinicopathologic features. As a result, we found C7/CFH/LSF-1-high tumors associated with larger tumor size (9.35 vs 4.6 cm, p=0.01827, Figure 7A) and interestingly, a tendency to develop in the background of normal appearing liver (Table 3).

4. Discussion

Our results here implicate the novel functions of the complement proteins in TICs. We enriched primary TICs from HCC using tumorsphere culture and inject them into mice that develop tumor. Then, we isolated and analyzed RNA to unravel novel TIC surface biomarkers and surprisingly found a significant increase in complement proteins.

Complement proteins are mainly produced in the liver and play a role in innate immunity by forming a pore in antigen-presenting cells [22]. However, recent reports suggest their function in tumor formation, growth, and metastasis. Cho et al [23] reported that tumor-derived complement protein 3 is secreted, comes back to the liver cells, and stimulates growth in ovarian tumors [23]. In addition, C3a and C5a affect the tumor microenvironment by controlling cytokines and growth factors such as interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF). These factors are known to enhance tumor cell invasion and migration by controlling the tumor microenvironment, including endothelial cells [24]. However, there are no reports that describe how complement proteins C7 and CFH function in TICs, especially for upregulating stemness. A recent report showed that CFH is upregulated in cutaneous squamous cell carcinoma (cSCC), and its knockdown inhibits the proliferation and migration of cSCC cells [25]. In contrast, the role of C7 in cancer cells is still not well understood. There are known receptors of complement C3a–C5a, and some are functionally characterized but mainly related to the innate immune system [26–28]. Therefore, it is unclear if C7 or CFH can be secreted and internalized into other cancer cells in order to function as a stemness regulator. Because the complement proteins C7 and CFH do not have either DNA-binding activities or transcription factor functions, we reason that certain mediators would receive a signal from these complement proteins and play a role in the transcriptional activation of stemness factor genes.

In order to find a such a factor, we searched the literature and found that the transcription factor LSF-1 transactivates CFH and is overexpressed in HCC [20]. Our data presented here revealed that the complement proteins C7 and CFH function via LSF-1 to increase stemness in liver TICs (Figure 7C). Of note, C7/CFH/LSF-1-high tumors were developed in normal appearing liver in our clinical sample analysis (Table 2). At present, it is not clear how the C7 and CFH can increase LSF-1 expression. As there is no known DNA binding or transcription factor function for C7 or CFH, the increase of LSF-1 might be through another mediator in nucleus. Nonetheless, our result suggests that complement-LSF-1 functional axis is possibly associated with *de novo* hepato-carcinogenesis from normal appearing

hepatocytes, instead of multistep carcinogenesis from cirrhosis-dysplastic nodule-carcinoma sequence (Table 2 and 3). We tested the expression levels of stemness factors along with LSF-1 in tumor specimens, but could not find a significant correlation (Supplementary Figure 7). This result could be a masking effect of differentiated tumor cells due to the small number of TICs in tumor tissue, where upregulated LSF-1 can upregulate stemness factors and, thereby, the cells acquire stemness. Further studies are needed to fully understand how intracellular C7 and CFH induce LSF-1 expression, as well as other unknown factors. These efforts will provide novel insights into the functions of complement proteins and their applications as biomarkers, as well as therapeutic targets, for TICs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69–90. [PubMed: 21296855]

2. Aravalli RN, Cressman EN, Steer CJ. Cellular and molecular mechanisms of hepatocellular carcinoma: an update. Arch Toxicol. 2013; 87:227–247. [PubMed: 23007558]

3. Visvader JE. Cells of origin in cancer. Nature. 2011; 469:314–322. [PubMed: 21248838]

4. Easwaran H, Tsai HC, Baylin SB. Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. Mol Cell. 2014; 54:716–727. [PubMed: 24905005]

5. Visvader JE, Lindeman GJ. Cancer stem cells: current status and evolving complexities. Cell Stem Cell. 2012; 10:717–728. [PubMed: 22704512]

6. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? Nature medicine. 2009; 15:1010–1012.

7. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. Annals of the New York Academy of Sciences. 2009; 1155:206–221. [PubMed: 19250206]

8. Singh SR. Cancer stem cells: recent developments and future prospects. Cancer Letters. 2013; 338:1–2. [PubMed: 23583678]

9. Bishayee A. The role of inflammation and liver cancer. Advances in Experimental Medicine and Biology. 2014; 816:401–435. [PubMed: 24818732]

10. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420:860–867. [PubMed: 12490959]

11. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010; 140:883–899. [PubMed: 20303878]

12. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet. 2008; 371:771–783. [PubMed: 18275997]

13. Fan QM, Jing YY, Yu GF, Kou XR, Ye F, Gao L, Li R, Zhao QD, Yang Y, Lu ZH, Wei LX. Tumor-associated macrophages promote cancer stem cell-like properties via transforming growth factor-beta1-induced epithelial-mesenchymal transition in hepatocellular carcinoma. Cancer Letters. 2014; 352:160–168. [PubMed: 24892648]

14. Peng Q, Li K, Anderson K, Farrar CA, Lu B, Smith RA, Sacks SH, Zhou W. Local production and activation of complement up-regulates the allostimulatory function of dendritic cells through C3a-C3aR interaction. Blood. 2008; 111:2452–2461. [PubMed: 18056835]

15. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. Nature Medicine. 2002; 8:582–587.

16. Raedler H, Yang M, Lalli PN, Medof ME, Heeger PS. Primed CD8(+) T-cell responses to allogeneic endothelial cells are controlled by local complement activation. American Journal of Transplantation. 2009; 9:1784–1795. [PubMed: 19563342]

17. Strainic MG, Liu J, Huang D, An F, Lalli PN, Muqim N, Shapiro VS, Dubyak GR, Heeger PS, Medof ME. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunity. 2008; 28:425–435. [PubMed: 18328742]

18. Vadrevu SK, Chintala NK, Sharma SK, Sharma P, Cleveland C, Riediger L, Manne S, Fairlie DP, Gorczyca W, Almanza O, Karbowniczek M, Markiewski MM. Complement c5a receptor facilitates cancer metastasis by altering T-cell responses in the metastatic niche. Cancer Research. 2014; 74:3454–3465. [PubMed: 24786787]

19. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, Coukos G, Lambris JD. Modulation of the antitumor immune response by complement. Nat Immunol. 2008; 9:1225–1235. [PubMed: 18820683]

20. Santhekadur PK, Rajasekaran D, Siddiq A, Gredler R, Chen D, Schaus SE, Hansen U, Fisher PB, Sarkar D. The transcription factor LSF: a novel oncogene for hepatocellular carcinoma. American Journal of Cancer Research. 2012; 2:269–285. [PubMed: 22679558]

21. Yoo BK, Emdad L, Gredler R, Fuller C, Dumur CI, Jones KH, Jackson-Cook C, Su ZZ, Chen D, Saxena UH, Hansen U, Fisher PB, Sarkar D. Transcription factor Late SV40 Factor (LSF) functions as an oncogene in hepatocellular carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:8357–8362. [PubMed: 20404171]

22. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. Frontiers in Immunology. 2015; 6:257. [PubMed: 26074922]

23. Cho MS, Vasquez HG, Rupaimoole R, Pradeep S, Wu S, Zand B, Han HD, Rodriguez-Aguayo C, Bottsford-Miller J, Huang J, Miyake T, Choi HJ, Dalton HJ, Ivan C, Baggerly K, Lopez-Berestein G, Sood AK, Afshar-Kharghan V. Autocrine effects of tumor-derived complement. Cell Rep. 2014; 6:1085–1095. [PubMed: 24613353]

24. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. Journal of Immunology. 2009; 182:4499–4506.

25. Riihila PM, Nissinen LM, Ala-aho R, Kallajoki M, Grenman R, Meri S, Peltonen S, Peltonen J, Kahari VM. Complement factor H: a biomarker for progression of cutaneous squamous cell carcinoma. The Journal of Investigative Dermatology. 2014; 134:498–506. [PubMed: 23938460]

26. Cravedi P, van der Touw W, Heeger PS. Complement regulation of T-cell alloimmunity. Seminars in nephrology. 2013; 33:565–574. [PubMed: 24161041]

27. Weiss L, Fischer E, Haeffner-Cavaillon N, Jouvin MH, Appay MD, Bariety J, Kazatchkine M. The human C3b receptor (CR1). Advances in Nephrology from the Necker Hospital. 1989; 18:249–269. [PubMed: 2522267]

28. Zaidi AK, Ali H. C3a receptors signaling in mast cells. Advances in Experimental Medicine and Biology. 2007; 598:126–140. [PubMed: 17892209]

Highlights

- C7 and CFH are required to maintain stemness in liver cancer cells

- Knockdown of C7 and CFH expression abrogated tumorsphere formation and induced differentiation

- Overexpression of C7 and CFH stimulated stemness factor expression as well as in vivo cell growth.

- Complement Proteins, C7 or CFH, are upregulated in liver TICs

- LSF-1 is induced by C7 or CFH and regulates stemness factor expression in liver TICs.
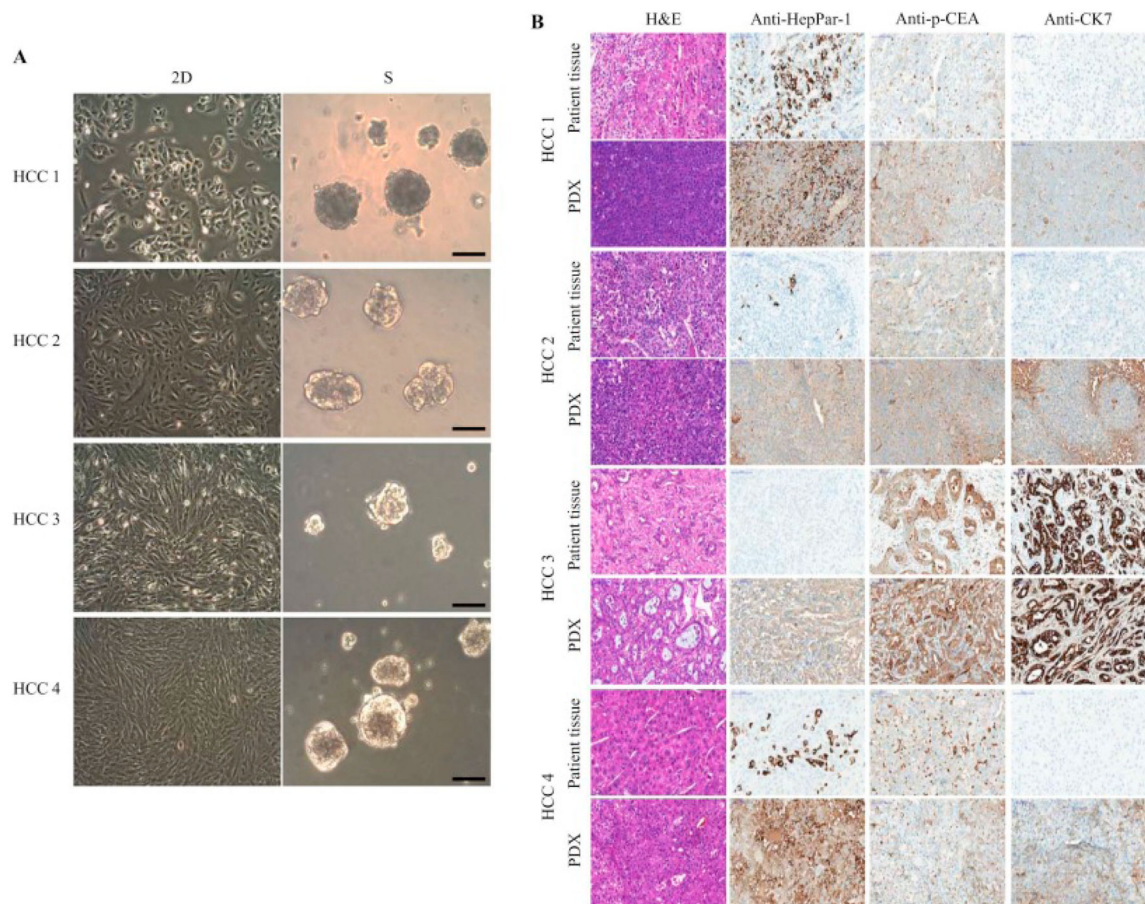
- C7, CFH, and LSF-1 are highly expressed in HCC tissues

Fig. 1.
Characterization of the tumorspheres and Patient Derived Xenografts (PDXs) derived from the 4 primary hepatocarcinoma (HCC) cells. (A) Representative pictures from tumorspheres derived from the primary HCC cells. 2D, 2D cultured; S, sphere; scale bar, 100μM. (B) Histological features of primary HCC and PDXs. Each of the tumors was stained with one of the H&E, Hep Par 1, p-CEA and CK7 antibodies.
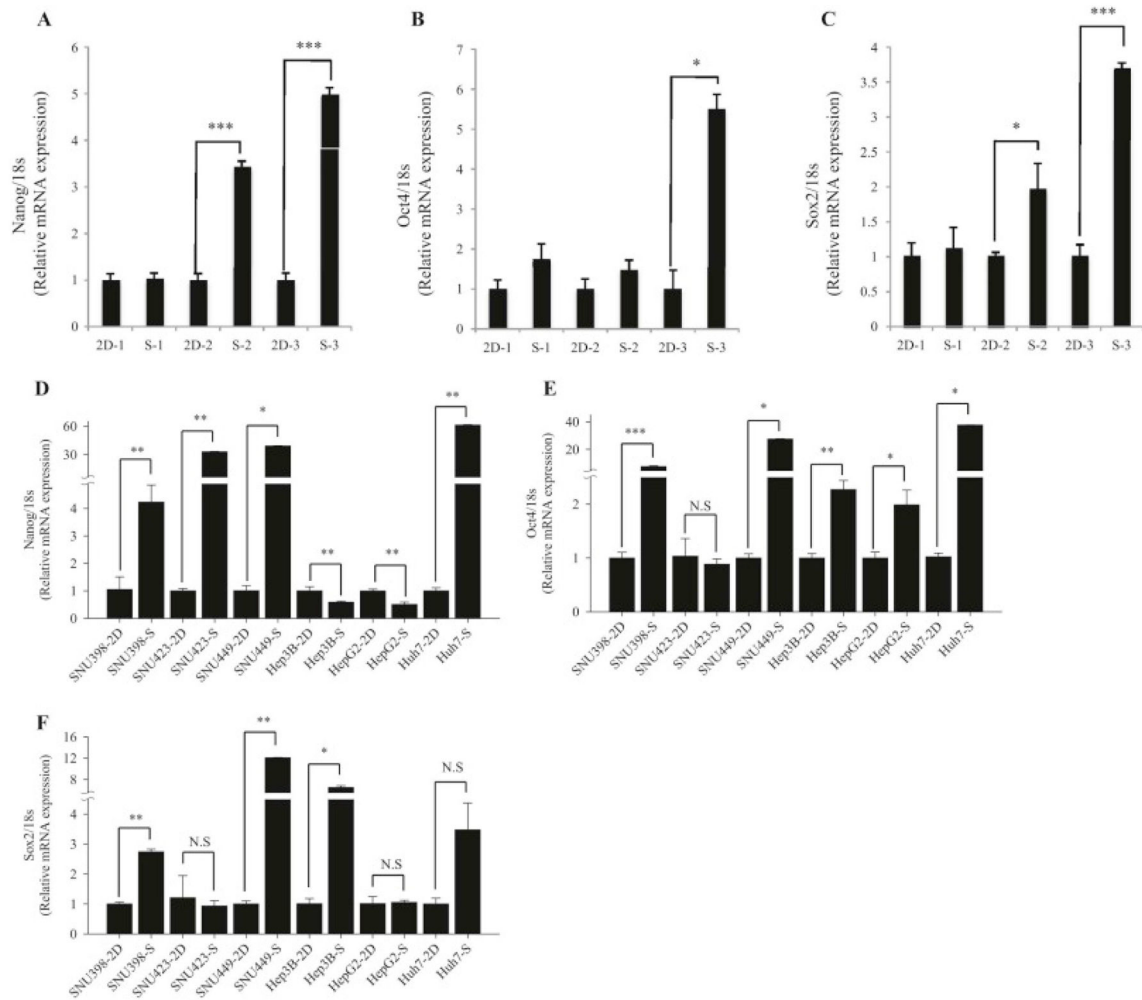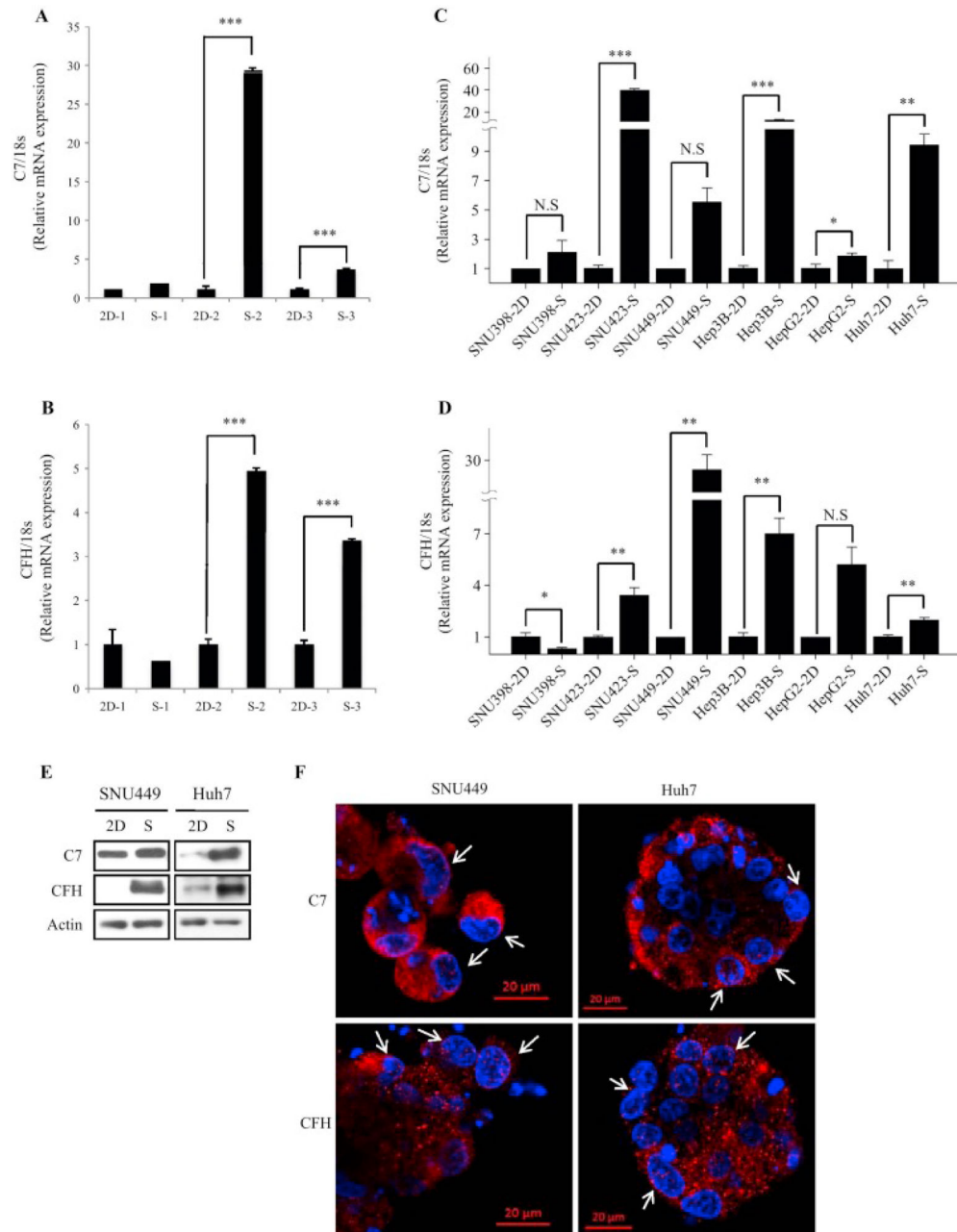
Fig. 2.
Tumorsphere cells expressed higher level of stemness factors than 2D cultured primary HCC cells. (A–C) Nanog (A), Oct4 (B) and Sox2 (C) RNA measurements from 2D and sphere (S) from primary HCC. (D–F) Nanog (D), Oct4 (E) and Sox2 (F) RNA measurements from 2D and sphere (S) derived from 6 liver cancer cell lines.

Fig. 3.
Complement protein C7 and CFH are highly expressed in the sphere (S) obtained from the primary HCCor immortalized cell lines. (A–B) C7 (A) and CFH (B) expressions in 2D and sphere (S) derived from primary HCC. (C–D) C7 (C) and CFH (D) expressions in 2D and sphere (S) obtained from liver cancer cell lines. (E) C7 and CFH protein levels in 2D and sphere (S) obtained from SNU449 and Huh7 cancer cells. Actin is used as the loading control. (F) Confocal microscopy for C7 and CFH protein localization in sphere (S) obtained from SNU449 and Huh7 cancer cells. Arrows indicate cells with C7 or CFH punctuate nuclear staining.
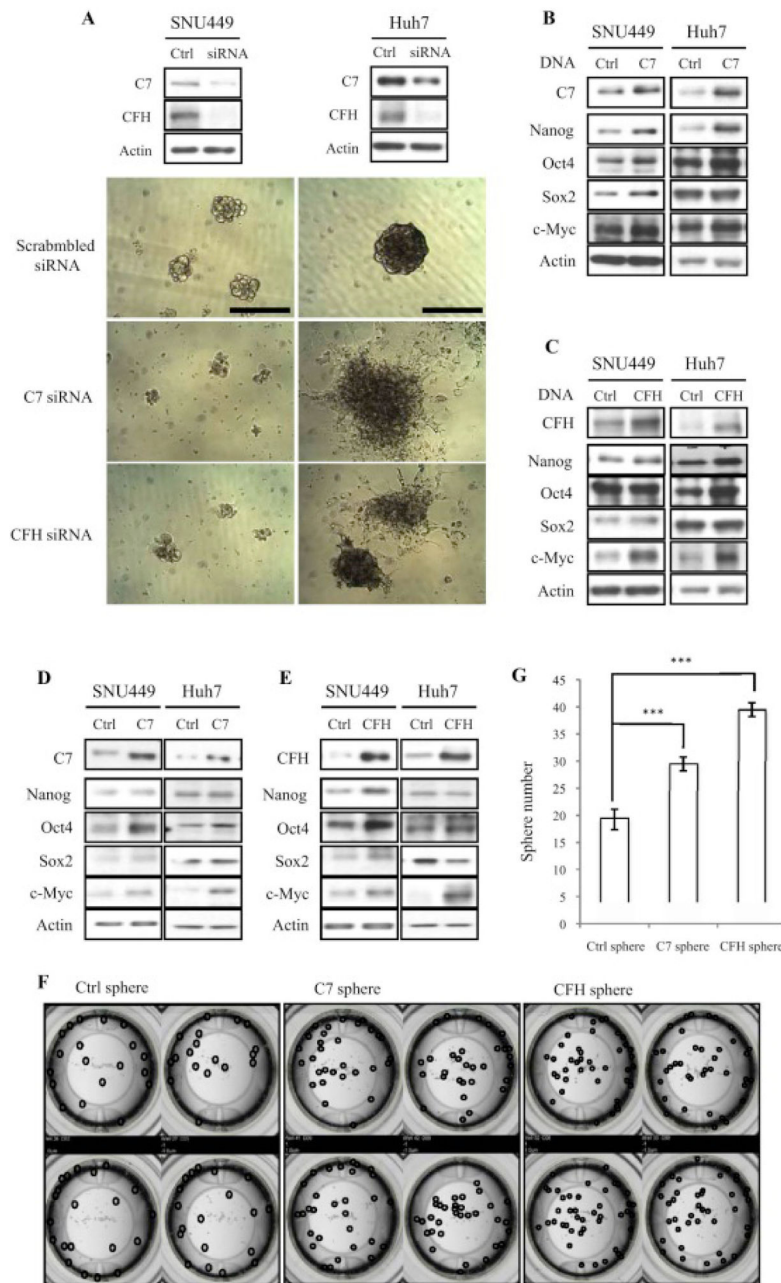
Fig. 4.
C7 and CFH are required for stemness factor expression in the liver cancer cell lines. (A) Morphological analysis of tumorspheres after C7 or CFH knockdown in SNU449 and Huh7 cells. The western blot shown shows the level of C7 and CFH proteins upon siRNA knockdown. Actin was used as a loading control. (B–C) Stemness factor protein analysis after transient overexpression of C7 (B) or CFH (C) in SNU449 and Huh7 cells. (D–E) Stemness factor expression in C7 (D) and CFH (E) stable cell lines generated from SNU449 and Huh7 cells. (F) Microscopic images of spheres obtained from C7 and CFH stable cell

lines (generated from Huh7 cells). (G) A Graph showing quantization of the spheres shown in (F).
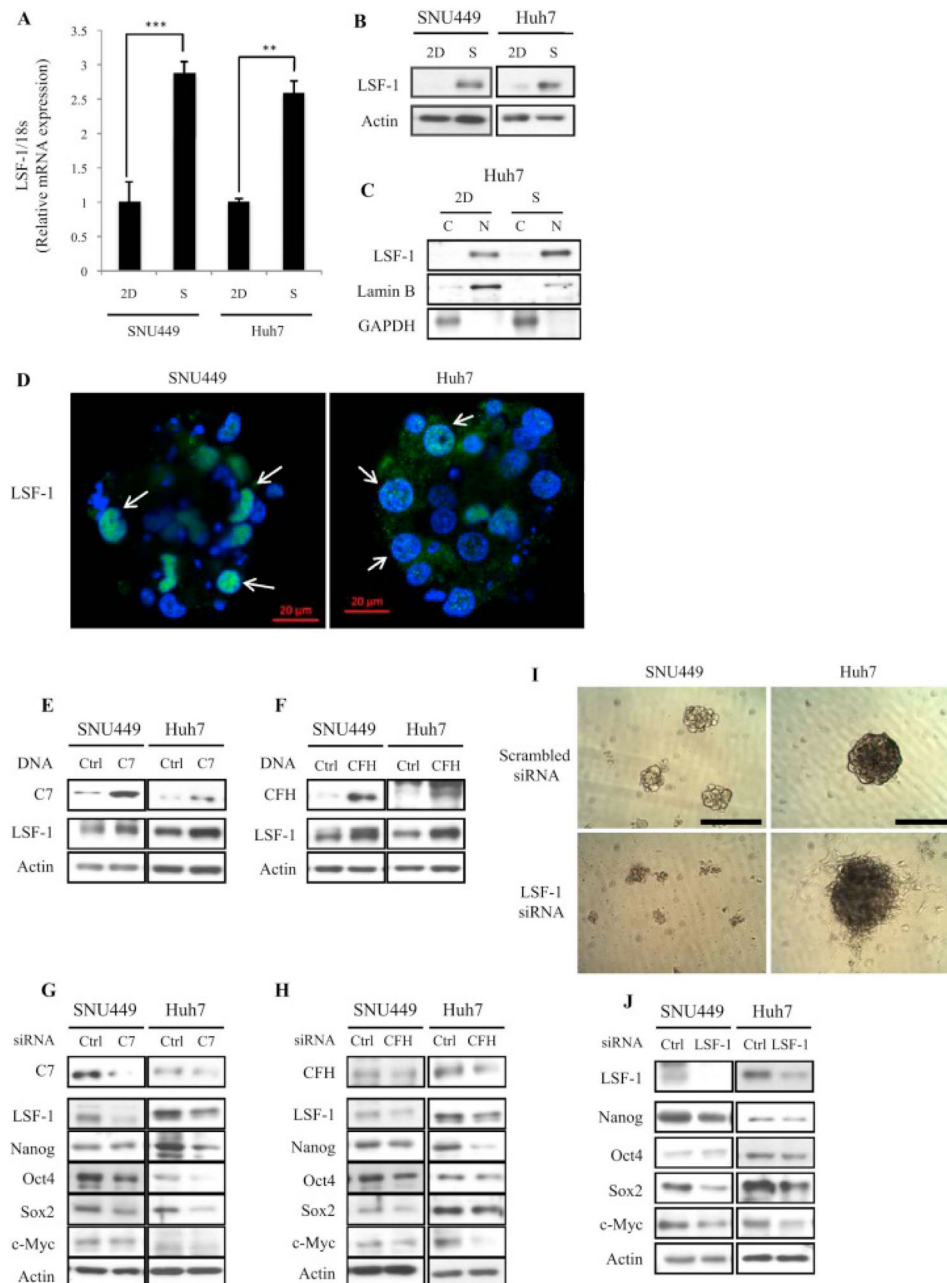
Fig. 5.
LSF-1 acts as a mediator between complement and stemness factor upregulation. (A–B) LSF-1 mRNA (A) and protein (B) expression in 2D and sphere (S) generated from SNU449 and Huh7 cells. (C) Western blot analysis of nuclear/cytoplasm fractionation of 2D and sphere (S) of Huh7. Lamin B and GAPDH were used as the fractionation controls for nuclear and cytoplasm, respectively. (D) Confocal microscopic images of LSF-1-immuno stained sphere obtained from SNU449 and Huh7 cells. Arrows indicate cells with LSF-1 punctate nuclear staining. (E–F) Western blot results showing LSF-1 protein expression in SNU449 and Huh7 cells under the condition of C7 (E) or CFH (F) overexpression. (G and
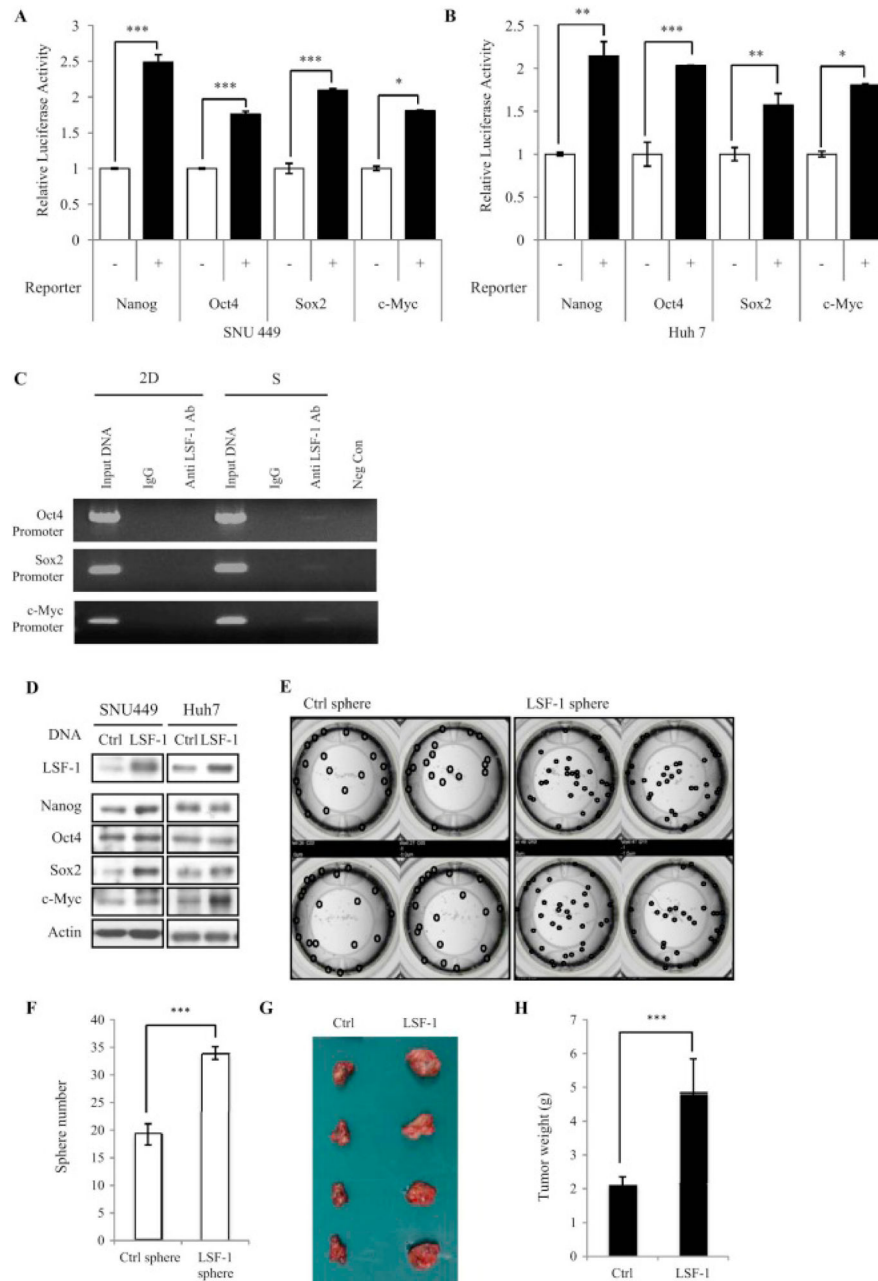
H) Western blot results for LSF-1 and stemness factor after treatment with 50 nM C7 (G) or CFH (H) siRNA in SNU449 and Huh7 cells. (I) Morphological changes in the tumorsphere after LSF-1 knockdown in SNU449 and Huh7 cells. Scrambled siRNA was used as the negative control. (J) Western blot results of the stemness factors after LSF-1 siRNA transfection in SNU449 and Huh7 cells.

Fig. 6.
Direct upregulation of Nanog, Oct4, Sox2, and c-Myc promoters by LSF-1 enhances tumorsphere formation and the *in vivo* growth of liver cancer cells. (A–B) Dual luciferase reporter assay with the pGL3-Nanog, Oct4, Sox2, and c-Myc promoter reporters. LSF-1 was co-transfected with each of the reporter vectors into SNU449 (A) and Huh7 (B) cells. (C) ChIP assay results for LSF-1 that was tested on the 3 promoter regions. ChIP was performed on SNU449 and Huh7 cells with antibodies for LSF-1 and normal mouse IgG, and the signal was detected using primers that targeted a part of the Sox2, Oct4, and c-Myc gene promoters. (D) Effect of LSF-1 overexpression on stemness factor expression. SNU449 and

Huh7 cells were transfected with control or LSF-1 expression vectors. The expression of stemness factors wereanalyzed using western blot analysis. (E) Effect of LSF-1 stable expression on tumorsphere formation in Huh7 cells. Representative pictures of the sphere in control (left) and LSF-1 stable cells (right). (F) A Graph showing quantification of the image in (E). (G) Picture showing the tumors dissected from mouse injected with control (Ctrl) or LSF-1 stable cells. (H) Measurements of tumor weight for the xenograftstumors shown in (G).
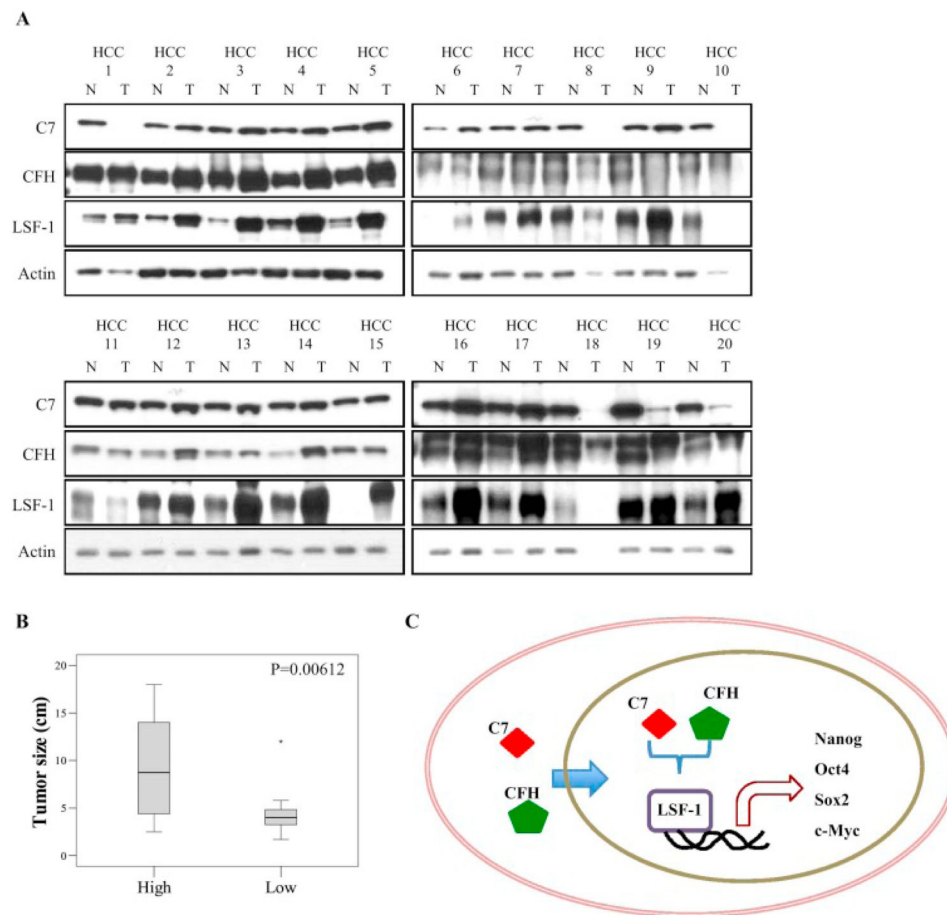
Fig. 7.
C7, CFH, and LSF-1 expression are correlated with tumor size in HCC patient specimens.
(A) Western blot of C7, CFH, and LSF-1 in HCC (T) and surrounding non-neoplastic (N)
liver specimens. (B) Correlation analysis of C7/CFH/LSF-1 expression and tumor size. High
or Low indicate samples with a higher or lower level of C7/CFH/LSF-1 in the tumor,
compared with the control pair. (C) Proposed molecular mechanism by which C7 and CFH
promote stemness in liver TICs. C7 and CFH stimulate LSF-1 expression, which is localized
in the nucleus and binds to the promoters of the Nanog, Oct4, Sox2, and c-Myc genes. This
leads to the upregulation of the stemness factors, which finally increases cancer stemness.

Table 1

Upregulated pathways in tumorsphere cultured hepatocellular carcinoma cells

Pathway	%	p-Value	Genes
Complement and coagulation cascades	63.2	1.60E-18	C7, C4A, C3, CFB, C4B, C6, CFH, C1R, SERPING1, C1S, C2, C8G
Systemic lupus erythematosus	47.4	8.90E-11	C7, C4A, C3, C4B, C6, C1R, C1S, C2, C8G
Prion diseases	15.8	4.60E-03	C7, C6, C8G
NOD-like receptor signaling pathway	15.8	1.40E-02	CCL11, CXCL1, CXCL2

Table 2

C7, CFH and LSF-1 expression and clinicopathologic features of hepatocellular carcinoma patients.

No.	Age	Gender	Tumor Size (cm)	Tumor Necrosis	Microvessel invasion	E-S Grade	Viral association	Nontumor liver parenchyma	Overexpression in Tumor		
									C7	CFH	LSF
1	44	M	1.7	N	N	1	NBNC	Cirrhosis	ND	N	Y
2	59	M	15	Y	N	2	NBNC	Minimal change	Y	Y	Y
3	41	M	8.5	Y	N	4	HBV	Minimal change	Y	Y	Y
4	44	M	13	Y	N	3	NBNC	Minimal change	Y	Y	Y
5	56	F	2.5	N	N	4	HCV	Cirrhosis	Y	Y	Y
6	44	M	3.5	N	N	3	HBV	Chroic hepatitis	Y	N	Y
7	43	M	4.7	N	N	3	HBV	Cirrhosis	Y	N	Y
8	51	M	5.8	Y	Y	4	HBV	Cirrhosis	ND	N	N
9	50	F	5	N	N	3	HBV	Cirrhosis	Y	N	Y
10	53	M	3	N	N	4	HBV	Cirrhosis	ND	N	ND
11	56	M	4.5	Y	N	3	HBV	Chroic hepatitis	N	N	N
12	71	F	9	N	N	3	HBV	Minimal change	Y	Y	Y
13	56	M	12	Y	Y	2	B&C	Chroic hepatitis	Y	N	Y
14	53	M	18	Y	N	3	NBNC	Minimal change	Y	Y	Y
15	63	M	4	N	N	3	HBV	Cirrhosis	Y	N	Y
16	57	M	4.8	Y	N	4	HBV	Minimal change	Y	Y	Y
17	42	F	4	N	N	2	HBV	Crrrhosis	Y	Y	Y
18	26	M	4	Y	N	3	HBV	Cirrhosis	ND	ND	ND
19	68	M	4	N	N	2	HBV	Cirrhosis	N	N	Y
20	46	M	3	N	N	4	HBV	Cirrhosis	N	N	Y

N: No, Y: Yes, ND: Not Detected, E-S Grade: Edmondson–Steiner Grade, HBV: Hepatitis B Virus, HCV: Hepatitis C Virus, NBNC: Non-B non-C Virus

Table 3

Correlation between C7/CFH/LSF-1 level and non-neoplastic parenchymal histology

C7/CFH/LSF-1	Ht or Ch	MC
High	2	6
Low	12	0

Ht: Hepatitis, Ch: Cirrhosis, MC: Minimal Change

p=0.0187 (Fisher's test)