Positive Feedback Loop of OCT4 and c-JUN Expedites Cancer Stemness in Liver Cancer

KUNG-KAI KUO,a,b KING-TEH LEE,a KER-KONG CHEN,c YA-HAN YANG,a,b,d YING-CHU LIN,c MING-HO TSAI,d KENLY WUPUTRA,d YEN-LIANG LEE,e CHIA-CHEN KU,d HIROYUKI MIYOSHI,f YUKIO NAKAMURA,e SHIGEO SAITO,h CHUN-CHIEH WU,i CHEE-YIN CHAI,l RICHARD ECKNER,j CHEN-LUNG STEVE LIN,a,d SOPHIE S-W WANG,b,k DENG-CHYANG WU,b,k,l CHANG-SHEN LIN,d,n KAZUNARI K. YOKOYAMAa,b,d,m,o,p,q

Key Words. Cancer stem cells • c-JUN • Liver cancer • OCT4 • Positive feedback • Pluripotency • Reprogramming

ABSTRACT

The network of stemness genes and oncogenes in human patient-specific reprogrammed cancer stem cells (CSCs) remains elusive, especially in liver cancer. HepG2-derived induced pluripotent stem cell-like cells (HepG2-IPS-like cells) were generated by introducing Yamanaka factors and the knockdown vector shTP53. They exhibited features of stemness and a higher tumorigenesis after xenograft transplantation compared with HepG2 cells. The cancerous mass of severe combined immunodeficiency (SCID) mice derived from one colony was dissected and cultured to establish reprogrammed HepG2-derived CSC-like cells (designated rG2-DC-1C). A single colony exhibited 42% occurrence of tumors with higher proliferation capacities. rG2-DC-1C showed continuous expression of the OCT4 stemness gene and of representative tumor markers, potentiated chemoresistance characteristics, and invasion activities. The sphere-colony formation ability and the invasion activity of rG2-DC-1C were also higher than those of HepG2 cells. Moreover, the expression of the OCT4 gene and the c-JUN oncogene, but not of c-MYC, was significantly elevated in rG2-DC-1C, whereas no c-JUN expression was observed in HepG2 cells. The positive-feedback regulation via OCT4-mediated transactivation of the c-JUN promoter and the c-JUN-mediated transactivation of the OCT4 promoter were crucial for promoting cancer development and maintaining cancer stemness in rG2-DC-1C. Increased expression of OCT4 and c-JUN was detected in the early stage of human liver cancer. Therefore, the positive feedback regulation of OCT4 and c-JUN, resulting in the continuous expression of oncogenes such as c-JUN, seems to play a critical role in the determination of the cell fate decision from iPSCs to CSCs in liver cancer.

SIGNIFICANCE STATEMENT

Reprogramming of cancer cells is an innovative model for study of cancer stem cells. Here we report our successful reprogramming of the hepatoblastoma HepG2 into induced pluripotent stem cell (iPSC)-like cells, which yielded high tumor-formation in immunocompromised mice. A cell line rG2-DC-1C derived from the xenograft tumors exhibited increased cell proliferation, colony formation, enhanced invasion and chemoresistance. The aggressive phenotype of rG2-DC-1C is resulted from upregulation of c-JUN oncogene, which was activated by OCT4 in a positive feedback manner. This finding suggests the positive circuit of OCT4/c-JUN plays a critical role in cell fate determination from iPSCs to cancer stem-like cells.

INTRODUCTION

Liver cancers are the third most common cause of cancer-related deaths worldwide [1]. The current standard practices for the treatment of liver cancers, such as surgical resection and chemotherapy, are not satisfactory because of early metastasis and further post-operative recurrence [2]. The latter two processes depend on the existence of cancer stem cells (CSCs), which are defined by their self-renewing capacity and by their ability to produce new tumors [3]. CSCs have an indefinite growth potential, and are critical drivers of tumor formation. These cells have been identified in various human cancers, including liver cancers, and, because of their importance in tumorigenesis, CSCs have emerged as promising new targets for cancer therapy.
The reprogramming of both mouse and human somatic cells into induced pluripotent stem cells (iPSCs) has been achieved by expressing combinations of defined factors (OSKM), such as OCT4, SOX2, KLF4, MYC, and NANOG [4–8]. This technique can drive somatic cells to acquire “unlimited self-renewal” and pluripotency. This process of conversion from somatic to pluripotent stem cells encompasses global epigenetic alterations and chromatin changes that regulate the transcriptional system [9, 10]. The reprogramming of somatic cells to iPSCs is a quite efficient process. The study of CSCs may allow the elucidation of the process of tumorigenesis and the exploration of therapeutic agents that target CSCs specifically, however, the success rate of reprogramming cancer cells into iPSCs is much lower [11]. To date, this obstacle has restrained the generation of CSCs, but the reason for this failure is not understood fully.

Several studies have reported that a deviated reprogramming process in normal somatic cells may result in undifferentiated dysplastic cells or tumor development [12]. In particular, the OCT4 gene, which encodes a POU-domain transcription factor, is associated with an undifferentiated state of cancer cells [13, 14]. Kumar et al. [15] reported that the OCT4-mediated dedifferentiation of tumor cells may play an important role during tumor progression. In another study, OCT4 was shown to be a crucial regulator of HCC [16]. OCT4 was also proposed as a biomarker of CSC-like cells [14, 17–19]. The knockdown of OCT4 resulted in apoptosis in breast CSC-like cells [20]. However, the precise function of OCT4 in CSCs remains unclear.

In this study, we investigated whether the reprogramming technique mentioned above can be used to generate CSCs from transformed cells. We reprogrammed HepG2 cells, which are derived from a well-differentiated hepatoblastoma [21], into HepG2-iPSC-like cells by transducing lentivirus-encoded OSKM and shTP53. HepG2-iPSC-like cells carried stem-cell characteristics, and the HepG2-iPSC-like cell-derived xenograft tumor cells (reprogrammed HepG2-derived cancer stem-like cells from one colony; termed rG2-DC-1C) exhibited enhanced tumor sphere formation, enhanced colony formation, stronger tumorigenesis in vivo, these data suggest that OCT4/c-JUN could become therapeutic targets to eliminate CSCs in liver cancers.

Materials and Methods

Cell Culture and Animals

HepG2, 293T, mouse hepatocyte iPSCs (iPS-Hep-FB/Ng/gfp-103C-1), SNL76/7, and AML12 cells obtained from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and ATCC (Manassas, Virginia, U.S.) were cultured in either Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Grand Island, New York, U.S., https://www.thermofisher.com/us/en/home/brands/gibco.html) or mouse iPS medium with mouse LIF (1000 U/ml). All media were supplemented with 10% FBS (Biological Industries; Kibbutz Beit Haemek, Israel) and 1% penicillin and streptomycin (Gibco). The animal experiments were approved by the Animal Care Committee of the RIKEN BioResource Center in Japan, the National Laboratory of Animal Center in Taiwan, and the Kaohsiung Medical University in Taiwan.

Generation of iPSC-like Cells and Establishment of Reprogrammed HepG2-Derived Cancer Cells

Recombinant lentiviruses including CSIV-CMV-MCS-RES2-Venus-encoded human OCT4, KLF4, SOX2, C-MYC (OSKM), c-JUN, shTP53, CSII-EF-Flag-WT-c-JUN-RES2-Venus or CSII-EF-Flag-c-JUN (constitutive active D-63/D-73)-RES2-Venus were generated using 293T cells that were cotransfected with pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev. HepG2, AML and iPS-Hep-FB/Ng/gfp-103C-1 (5 × 10⁴) were infected with lentiviruses (MOI = 2) and incubated for 1 week in DMEM. Thereafter, cells were transferred onto mitomycin C-treated mouse embryonic fibroblasts in DMEM plus ESGRO (10 ng/ml) to obtain reprogrammed HepG2-derived iPSC-like cells. One colony (approximately 200 cells) of HepG2-derived iPSC-like cells was injected into severe combined immunodeficiency (SCID) mice and tumor was formed 9 weeks later. The tumor was resected to establish a primary cancer cell line designated as “reprogrammed HepG2-derived cancer cells from one colony” or “rG2-DC-1C”.

Teratoma Formation and Immunohistochemistry

HepG2-iPSC-like cells (200 cells; one colony) were injected subcutaneously into severe combined immunodeficiency mice and the teratomas were examined by immunohistochemistry as described [22].

Alkaline Phosphatase, Immunocytochemistry, and Sphere- and Colony-Formation Assays

These experiments were performed as described [22, 23].

Chemicals, Antibodies, and Western Blotting

The chemicals used were: SB202190 (p38 inhibitor; Millipore, Billerica, Massachusetts, U.S.), SP600125 (JNK inhibitor; Biomol International–Enzo, Kelayres, Pennsylvania, U.S.), U0126 (MEK inhibitor; Millipore), and FR180204 (ERK inhibitor; Millipore). Antibodies were from Cell Signaling (Dancers, Massachusetts, U.S.): OCT4 (2750), c-JUN (60A8), p-c-JUN-Ser63 (7290), and -ERK (14799), p-ERK (14794); Stemgent (Cambridge, Massachusetts, U.S.): SSEA-3 (09-0014), SSEA-4 (090006); Millipore: SOX2 (AB5603), Tra-1-60 (MAB4360A4), Tra-1-81 (MAB4381A4), GAPDH (MAB374), ABCG2 (OP191); Santa Cruz (Dallas, Texas, U.S.): KLF4 (sc-20691), Brd4 (sc-48872), p21 (sc-397), CCND3 (sc-182), JNK (sc-571), p-JNK (81E11); Stemgent (Cambridge, Massachusetts, U.S.): SSEA-3 (09-0014), SSEA-4 (090006); Millipore: SOX2 (AB5603), Tra-1-60 (MAB4360A4); Tra-1-81 (MAB4381A4), GAPDH (MAB374), ABCG2 (OP191); Santa Cruz (Dallas, Texas, U.S.): KLF4 (sc-20691), Brd4 (sc-48872), p21 (sc-397), CCND3 (sc-182), JNK (sc-571), β-actin (sc-81178); Abgent (San Diego, California, U.S.): AFP (AJ019a), ABCB1 (AP6111a); and BD Biosciences (San Jose, California, U.S.): E-cadherin (610404). Western blot was performed as described [22–24].
Cell Growth, BrdU Incorporation, and Cell-Cycle Analyses
Cell growth was examined by counting the living cells stained with trypan blue at the indicated times. The cells in S phase were labeled using the BrdU Labeling and Detection Kit I as described [23]. For analysis of the cell cycle, serum-starved cells were cultured in DMEM containing 15% FBS and collected at the indicated times. Harvested cells were stained with propidium iodide (1 μg/ml), and subjected to flow cytometric analysis (EPICS XL-MCL; Beckman Coulter, Miami, Florida, U.S.).

Invasion Assay
Cells (1 × 10⁵ cells) were seeded on Transwell coated with Matrigel (Corning, Corning, New York, U.S. 1 mg/ml) without serum. The Transwell was then put on a plate contained DMEM plus 10% FBS for three days. The invaded cells on the lower surface of membrane were fixed, stained, and counted under a microscope according to manufacturer’s instruction.

Chemoresistance
Cells (5 × 10³) in 96-well plates were treated with indicated concentrations of 5-fluorouracil, cisplatin, and doxorubicin for 48 hours and then cell viability was examined by MTT assay.

RNA Sequencing and Gene Clustering
RNA sequencing was performed by Welgene Biotech (Taipei, Taiwan) according to the manufacturer’s protocol (Illumina, San Diego, California, U.S.). The cDNA libraries were prepared using TruSeq RNA Sample Prep kits and were sequenced on an Illumina GAIIx platform. Raw sequences were obtained using the CASAVA Pipeline software and the low-quality data were trimmed by ConDeTri. After filtering, qualified reads were analyzed using TopHat/Cuffdiff for gene expression estimation. The Human Genome Build 19 and gene features were used for data processing. The gene expression level was calculated as fragment/kilobase of transcript/million mapped reads (FPKM). Differentially expressed genes were filtered by FPKM ≥ 0.3, fold change ≥ 2, and p value < .05. Gene-level normalization was performed by transforming the FPKM/gene/sample to a Log2 median-centered ratio. Clustering was performed using Euclidean distance and complete linkage settings. Heatmap was generated by coloring each gene on the Log2 median-centered ratio. Lists of liver cancer genes, oncogenes, tumor suppressor genes, stemness genes, and OCT4-signaling-related genes were collected from GSEA, KEGG, GeneOntology, Life Technology panels, or Qiagen panels. The RNA sequencing data were deposited in the NCBI Bioproject Database with the accession number PRJNA273617.

Luciferase Assay
rG2-DC-1C or HepG2 (1 × 10⁵ cells) were plated onto each well of 12-well plates and cultured for 24 hours. The cells were then cotransfected with indicated amounts of effecter plasmids expressing OCT4, c-JUN, or dominant-negative mutants of c-JUN (S63A or S73A), together with firefly luciferase reporters carrying the wild type or various mutants of OCT4 or c-JUN promoters. The pGL4 plasmid encoding Renilla luciferase (Promega, Madison, Wisconsin, U.S.) was included to calibrate transfection efficiency. The total amount of transfected DNA was kept constant at 1 μg/well. After 48 hours or as indicated times, the cells were harvested and the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wisconsin, U.S.) and an illuminometer (Berthold, Bad Wildbad, Germany).

Chromatin Immunoprecipitation (ChIP) Assay
ChIP assays were performed as described [23, 24]. The primers are shown in Supporting Information Table S1.

shRNA and siRNA
The OCT4 shRNA construct (TRCN0000004881) was obtained from Academia Sinica, Taipei, Taiwan. The siRNAs specific for c-JUN (S7658), E-cadherin (S27768), and negative control (A390843) were purchased from Thermo Fisher (Waltham, Massachusetts, U.S.).

Tumor Formation by Recombinant Viruses Encoding OCT4 And c-JUN
Primary mouse hepatocytes were prepared and infected with c-JUN adenovirus (RDB02762, RIKEN DNA Bank, Tsukuba, Japan; MOI = 5) and/or OCT4 lentivirus (MOI = 10). The infected primary hepatocytes (1 × 10⁵ and 1 × 10⁶) were injected into both sides of mouse backs, and then the mice were weekly inspected using the IVIS200 Imaging System with Living Image software, version 4.2 (Caliper Life Sciences, Hopkinton, Massachusetts, U.S.). Tumor size was measured using a caliper, once or twice a week, and was estimated according to the formula: volume (cm³) = (L × W²)/2, where L and W were the length and width of the tumor, respectively. The mouse survival rates were estimated using Kaplan–Meier method with log-rank test.

Patient Samples
This study was approved by the Institutional Review Board of the Kaohsiung Medical University Hospital (KMUH, IRB-960343). The patients with hepatocellular carcinoma were enrolled between June 2010 and August 2013 with written informed consent. At the time of surgery, all tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Statistical Analyses
Data are presented as the mean ± SEM (n = 3 or more as indicated). One-way ANOVA or two-tail Student’s t-tests were used to examine statistical significance. A p-value < .05 was considered significant.

RESULTS
Although the coordinate ectopic expression of OSKM induces the reprogramming of somatic cells to iPSCs [6], the activation of individual factors can sometimes contribute to tumorigenesis [11, 12]. Moreover, cancer cells are reprogrammed very inefficiently, and only a subset of cancers are amenable to reprogramming [11]. To obtain reprogrammed cancer-specific iPSCs, we generated iPSCs from the human HepG2 (hepatoblastoma) and Huh-7 (hepatocellular carcinoma) cell lines by introducing recombinant lentiviruses encoding OSKM, together with the shTP53 knockdown lentivirus. After culturing cells in the presence of the leukemia inhibitor factor in medium for 30–40 days, we isolated iPSC-like colonies and cultured them for additional 2 weeks to generate iPSC-like cells (Fig.

www.StemCells.com
1A). These colonies comprised small, rapidly dividing cells with a high nuclear/cytoplasm ratio and large nucleoli. The reprogramming efficiency was 0.5% for HepG2 cells. However, we did not obtain any colony from Huh-7 cells for unknown reasons. Stem cell characteristics were verified by immunofluorescence staining of alkaline phosphatase (Fig. 1B; Supporting Information Fig. S1A), TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4 (Fig. 1C). However, HepG2 cells did not show such staining (data not shown). The HepG2-iPSC-like cells were injected into SCID mice to examine teratoma formation; these cells differentiated preferentially into two germ layers, endoderm and mesoderm (Fig. 1D). The expression of ectoderm markers was not detectable. The HepG2-formed tumors were featured as endoderm (Fig. 1D, left). Thus, our HepG2-iPSC-like cells were not fully competent stem cells with a capacity to differentiate into three germ layers.

To examine the tumorigenicity of HepG2-iPSC-like cells in vivo, we injected iPSC-like colonies into SCID mice and found that all mice generated tumors after injection of 50 (approximately 10,000 cells) or 10 (approximately 2,000 cells) colonies (100%; Fig. 1E). The tumor-formation frequency by inoculation of one HepG2-iPSC-like colony (1C = ~200 cells) was still significant (41.7%; Fig. 1E, 1F). Mice injected with 200 HepG2 cells (the same cell number as one colony of HepG2-iPSC-like cells) showed no tumor formation (data not shown). Poorly differentiated cells with prominent neovascularization and hemorrhage associated with multinuclear giant cells were identified in the tumor (Fig. 1G). These results suggest that HepG2-iPSC-like cells are highly tumorigenic in vivo.

The tumors generated by one colony of HepG2-iPSC-like cells were dissected to establish rG2-DC-1C (i.e., reprogrammed HepG2-derived cancer cells from one colony) (Fig. 1A). The sphere-formation ability (an index for CSCs [25]) of rG2-DC-1C was 5.5-fold higher than that of the original HepG2 cells (Supporting Information Fig. S1B). The colony-formation ability of rG2-DC-1C was also 3.2- to 5-fold higher than that of HepG2 cells (Supporting Information Fig. S1C). For stemness gene expression, rG2-DC-1C cells showed significant elevation of OCT4 protein and a 2.5-fold increase in KLF4, whereas the levels of c-MYC and SOX2 were not changed (Fig. 2A). These data demonstrate that rG2-DC-1C cells exhibit stemness features that are similar to their parent HepG2-iPSC-like cells.
To examine the growth factor dependence, the proliferation ability of rG2-DC-1C and HepG2 cells was tested in the presence of various serum concentrations. A trypan blue dye-exclusion assay showed that rG2-DC-1C cells grew faster than did HepG2 cells under 5%, 1%, and 0.5% FCS (Fig. 2B), suggesting that rG2-DC-1C cells are more resistant to growth stress than are HepG2 cells. A representative cell-cycle analysis showed that 35%–37% of rG2-DC-1C cells were in the S phase, whereas only 22%–24% of HepG2 cells were in this phase (Fig. 2C), supporting the growth advantage of rG2-DC-1C cells.

A Transwell-invasion assay demonstrated that the invasion efficacy of rG2-DC-1C cells was nine-fold higher than that of HepG2 cells.

Figure 2. Features of rG2-DC-1C cells. (A): The expression of stemness proteins in rG2-DC-1C and HepG2 cells was examined by western blotting (30 μg protein per lane). (B): Proliferation of rG2-DC-1C and HepG2 cells. Cells were starved in medium containing 0.1% FCS for 24 hours and were then replated in medium containing 5%, 1.0%, or 0.5% FCS for 3 days. The mean number of cells in five independent plates was determined. A representative assay is shown (trypan blue dye-exclusion assay). (C): Cell cycle analysis. Serum-starved cells (5 × 10⁵) were cultured in medium containing 10% FCS for 18 hours, stained with PI, and then subjected to flow cytometry. (D): Invasion ability. The percentage of cell motility was the ratio of the number of invaded cells vs the total number of inoculated cells. (E): The drug-resistance capacities of HepG2 and rG2-DC-1C cells were demonstrated by the survival rates of cells treated with 5-fluorouracil (5-Fu), cisplatin (Cis), and doxorubicin (Dox). (F): The expression of ABCG2 in rG2-DC-1C and HepG2 cells was examined by immunofluorescent assay (magnification ×200). The expression of OSKM was shown as Venus (in yellow) and DNA was stained by DAPI (in blue). (G): Western blot analyses of ABCG2 and ABCB1 protein expression (30 μg protein per lane). Data in B, D and E are presented as means (n = 3) ± SEM, two-tailed Student’s test, *, p < .05; **, p < .01.
HepG2 cells (Fig. 2D), indicating that rG2-DC-1C cells have invasion features. To examine the drug-resistance ability of rG2-DC-1C cells, their viability was measured by incubating cells with a mixture of three chemotherapeutic drugs: 5-fluorouracil (0.25–20 μg/ml), cisplatin (0.5–40 μg/ml), and doxorubicin (0.125–10 μg/ml). The results showed that rG2-DC-1C cells were more resistance to these drugs than did HepG2 cells (Fig. 2E). The expression of ABCG2 and ABCB1 was enhanced by 2- to 3.5-fold in rG2-DC-1C compared with HepG2 cells (Fig. 2F, 2G), which supports the high-chemoresistance feature of rG2-DC-1C cells. Together, these data suggest a CSC characteristic for rG2-DC-1C cells.

To gain insight into the acquired CSC features of rG2-DC-1C cells by mean of changes in gene expression, we performed an RNA sequencing analysis. When rG2-DC-1C cells was compared with HepG2 cells, 26 upregulated genes (FPKM > 4.0) that are involved in tumor growth and CSC characteristics and 41 downregulated genes (FPKM < −4.0), some of which contribute to cell-cycle arrest, were observed in rG2-DC-1C cells (Supporting Information Fig. S2A). Because OCT4 was highly expressed in rG2-DC-1C cells, the differential expression of OCT4-regulated genes was selected (Fig. 3A). The genes upregulated in rG2-DC-1C cells included CCND3, BMPR1B, PRKCH, PPP2R2C, ID2, JUN, and TCF7. The downregulated genes were CD44, TIK3R3, LIFR, and SMAD5. Among these genes, c-JUN is particularly notable because it has been reported to be involved in the maintenance of self-renewal and tumorigenicity of glioma stem-like cells [26]. The results of Western blot confirmed an overexpression of c-JUN oncoprotein in rG2-DC-1C cells (Fig. 3B) and knockdown of OCT4 resulted in c-JUN downregulation (Fig. 3C), supporting that OCT4 is required for the upregulation of c-JUN in rG2-DC-1C cells. Moreover, Ingenuity Pathway Analysis using Yamanaka factors and TP53 showed a network connection between OCT4 and c-JUN (Supporting Information Fig. S2B). Taken together, it suggests that elevated expression of OCT4 and c-JUN may play a critical role in the CSC features of rG2-DC-1C cells.

By examining the TESS and TRANSFAC databases (http://www.gene-regulation.com/pub/databases.html), four putative OCT-binding sites were identified on the c-JUN promoter (Fig. 3D). The in vivo interaction between OCT4 and the c-JUN promoter was validated by a chromatin immunoprecipitation–qPCR assay. The results showed that OCT4 was recruited to the four OCT-binding sequences, mainly at the P4 (11-fold) and the P2 (56-fold) sites in rG2-DC-1C, but not in HepG2 cells (Fig. 3D). Control IgG did not recruit OCT4 to these sites. To validate these findings, c-JUN promoter–luciferase constructs containing mutations at OCT-binding sites were examined for transactivation by OCT4 in rG2-DC-1C cells. The basal activity of the 1,050 bp c-JUN promoter was higher in rG2-DC-1C cells than it was in HepG2 cells (data not shown). When the P2 or P4 OCT-binding site was mutated, the basal c-JUN promoter activity was decreased by ~50% (Fig. 3E) and, notably, the OCT4-mediated transactivation of c-JUN promoter was significantly impaired (Fig. 3F). Thus, the P2 and P4 OCT-binding sites are critical for transactivation of the c-JUN promoter by OCT4.

In colon cancer, c-JUN promotes a subpopulation of colorectal cancer cells to adopt a stem-cell like phenotype via the NANOG promoter [27, 28]. A recent study also showed that c-JUN upregulates the promoters of several stemness genes, such as OCT4, SOX2, and NANOG [29]. Thus, c-JUN might activate stemness genes in rG2-DC-1C cells via transactivation of the OCT4 promoter through a feedback mechanism. To address this hypothesis, we examined the c-JUN-mediated transactivation of OCT4 promoter–luciferase constructs containing three AP-1 sites (Supporting Information Fig. S3A). The results showed that the OCT4 promoter-Del-1 containing the proximal AP-1 site (P1, −116 to −109 relative to the transcriptional start site) only exhibited the highest basal activity, while mutation at this AP-1 site (mDel-1) reduced OCT4 promoter activity significantly (Supporting Information Fig. S3A). The c-JUN-mediated transactivation of OCT4 promoter-Del-1 were more profound than those of OCT4 promoter-Del-2 and -Del-3 (Supporting Information Fig. S3B). However, c-JUN-mediated transactivation of the OCT4 promoter-Del-1 (mDel-1) was largely impaired (Supporting Information Fig. S3B). Thus, the AP-1 site at P1 position is critical for the c-JUN-mediated transactivation of the OCT4 promoter. The role of the c-JUN-mediated activation of the OCT4 promoter was further supported by the analysis of c-JUN phosphorylation mutants at S63, S73, or both sites, which did not activate the OCT4 promoter in rG2-DC-1C (Supporting Information Fig. S3C). Moreover, the JNK inhibitor JP600125 and the ERK inhibitor FR182040, but not the p38MAPK inhibitor SB203580, decreased the OCT4 promoter activity in rG2-DC-1C cells (Supporting Information Fig. S3D). Western blot analysis confirmed that phosphorylated JNK and ERK were increased in rG2-DC-1C cells (Supporting Information Fig. S3E). These data suggest that c-JUN is involved in the activation of OCT4 promoter and that OCT4 is recruited to the c-JUN promoter, thus generating a positive signaling circuit between OCT4 and c-JUN.

Next, we examined the roles of OCT4 in cell proliferation, invasiveness, and chemoresistance by knockdown of OCT4 in rG2-DC-1C cells. The results showed that OCT4 knockdown in rG2-DC-1C cells decreased cell proliferation (Fig. 4A), BrdU incorporation (Fig. 4B), colony formation (Fig. 4C), and resistance to 5-fluorouracil, cisplatin, and doxorubicin (Fig. 4D). The results of invasion assay demonstrated that rG2-DC-1C cells carrying shRNA-OCT4 showed reduced invasive cells from 20 to 0.5% (Fig. 4E), but the control shRNA-HOXA13 did not (data not shown). Compared with the control shRNA, shRNA-OCT4 lentiviruses-transduced rG2-DC-1C cells showed a significant reduction of tumor formation in SCID mice (Fig. 4F). These results suggest that OCT4 is required for the enhanced CSC features observed in rG2-DC-1C cells.

To examine whether combined expression of c-JUN and OCT4 promotes tumorigenicity, we inoculated c-JUN adenovirus- and OCT4 lentivirus-infected mouse primary hepatocytes (10⁵ to 10⁶ cells) into SCID mice. The infected hepatocytes gave rise to tumors with an efficiency of 30%–40% (Fig. 5A), whereas hepatocytes infected with a single virus (either OCT4 or c-JUN) or the dominant-negative c-JUN plus OCT4 viruses did not form any tumors (data not shown). Mice that received primary hepatocytes infected with both c-JUN and OCT4 viruses showed low survival rate than did mice that received single-virus-infected hepatocytes (Fig. 5B). Moreover, we examined the effect of c-JUN or OCT4 on the mouse hepatocyte cell line AML12 and on mouse hepatocyte iPSC (IPS-Hep/FB/Ng/gfp-103C-1). The forced expression of c-JUN or OCT4 in these cells enhanced the expression of OCT4 or c-JUN significantly (Fig. 5C; Supporting Information Fig. S4A), followed by an increase in the invasion potency by approximately 2.0-to 5.0-fold (Fig. 5D; Supporting Information Fig. S4B). These results indicate that the continuous expression loop of OCT4 and c-JUN is critical for liver cancer.
development and that both c-JUN and OCT4 seem to form a positive feedback loop.

The expression of OCT4 was examined by immunohistochemistry in HCC specimens and adjacent normal tissues. OCT4 was detected in 78% (39/50) of HCC specimens (Table 1) and in only 10% (5/50) of normal liver tissues (Fig. 6A). The statistical correlation between OCT4 expression and HCC clinicopathological characteristics was analyzed; the results show a significant correlation between OCT4 expression and tumor size and number (Table 1). The c-JUN/AP-1 transcription factor is also a key component of many signal transduction pathways, including those involved in proliferation, differentiation, apoptosis, cell migration, and transformation [30]. Because an OCT4/c-JUN positive regulation was identified in this study, we also examined the expression of OCT4 in rG2-DC-1C cells. The c-JUN promoter constructs were transfected into rG2-DC-1C cells. The luciferase activities were measured 48 hours later. Data are presented as means (n = 3, 6, and 5 for D, E, and F, respectively) ± SEM (*, p < .05, **, p < .01).

![Figure 3. Identification of OCT4-mediated c-JUN expression in rG2-DC-1C cells.](image-url)
of c-JUN in HCC specimens. c-JUN was expressed in 46% (23/50) of HCC samples and was not present in 91% (10/11) of OCT4-negative HCC specimens (Fig. 6B). Among cases with positive OCT4 staining, 56% (22/39) of HCC specimens were also positive for c-JUN staining (Fig. 6C and Table 1). The correlation between the expression of OCT4 and c-JUN was statistically significant.

DISCUSSION

In cancer, stem cells are critical for carcinogenesis and resistance to anticancer treatment. The stemness gene OCT4, together with SOX2 and NANOG, maintains self-renewal and pluripotency. Conversely, OCT4 is also being regarded increasingly as a promising tumor biomarker for the diagnosis of germ cell tumors [31]. Recently, it was reported that OCT4 is detected in many somatic cancer cells from the esophagus, bladder, lung, liver, brain, and pancreas [13–19, 31–33]. The ectopic expression of OCT4 in normal breast cells led to the generation of cells with tumor-initiating and colonization abilities [32]. Moreover, ectopic expression of OCT4 enhanced the features of CSCs in a mouse model of breast cancer [33]. Overall, these studies highlight the importance of sustaining OCT4 expression in tumors for the maintenance of tumorigenic stem-cell-like characteristics. In this study, we successfully reprogrammed human cancer cells line (HepG2) to HepG2-iPSC-like cells, which exhibited the stemness feature. We also found that OCT4 was significantly upregulated in rG2-DC-1C cells, with no detectable expression of the OCT4 protein in HepG2 cells (Fig. 2A). Both HepG2-iPSC-like cells and rG2-DC-1C cells demonstrated the poorly differentiated HCC phenotype (Fig. 1G, data not shown).

It has been reported that the distal enhancer region of the OCT4 promoter is highly methylated in HepG2 cells compared with embryonic stem cells [34]. Instead of the full-length OCT4 protein, the OCT4B and OCT4B1 proteins derived from spliced forms of OCT4 are present in HepG2 cells [34]. However, we did not detect such altered forms of OCT4 or the complete OCT4 protein via western blotting (Fig. 2A).

After direct reprogramming using four factors, we found that only endogenous OCT4 was overexpressed because the exogenous RNA for OCT4 and lentivirus promoter was not detected and shut down in rG2-DC-1C cells (Fig. 2A, data not shown), which might have led to a reactivation of the core pluripotency autoregulatory circuitry. This loop can maintain pluripotency independent of the exogenous factors [35]. Recently, Ohnishi et al. [12, 36] reported the premature termination of reprogramming in somatic cells without any DNA defects, which caused cancer development, and demonstrated that the failed-reprogramming-associated somatic cells behaved similarly to cancer cells via altered epigenetic regulations. In an Oct4-inducible mouse model, forced expression of OCT4 in vivo blocked cell differentiation and caused severe epithelial
dysplasia associated with frequent mitotic figures, which mimics adenocarcinoma in situ [37]. OCT4 regulates the motility of bladder cancer cells and enhances tumor progression [38]. Another study showed that the overexpression of OCT4 led to chemoresistance via the activation of the AKT and ABCG2 pathways [39]. These previous works demonstrate that OCT4 may be required for the development and maintenance of the characteristics of CSCs. Here, we report that c-JUN and OCT4 maintained the oncogenic features of cancer stem-like cells derived from a single colony of HepG2-iPSC-like cells.

AP-1 transcription factors are expressed in most cell types, and are activated by oxidative stress, tumor promoters, or oncogene overexpression [40]. These previous studies provide indirect evidence that AP-1 is an important key regulator of cancer cell growth and invasion [41, 42]. Here, we found that the constitutive increase in OCT4 induced oncogenic features via the activation of the c-JUN promoter (Figs. 3F, 6D), which supports a role for AP-1 in the regulation of cell growth and invasion. Besides, increased c-JUN phosphorylation at serine residues 63 and 73 is critical for the induction of OCT4

Figure 5. Critical roles of OCT4 and c-JUN in liver tumor formation and invasion in mouse primary hepatocytes- and hepatocyte-specific iPSCs. (A): Tumor-formation ability of mouse primary hepatocytes (10^4–10^6 cells) infected, alone or in combination, with the OCT4 lentivirus (MOI = 10) and the c-JUN adenovirus (MOI = 10), which were introduced via xenograft transplantation. Ten (single virus infection) or 22 (combined virus infection) mice were used to calculate the tumor formation efficiencies. (B): Kaplan-Meier plot showing the survival probability of mice in each treatment group (log-rank test). The vector control did not show any tumor formation by 120 days (data not shown). (C): Mouse hepatocyte-specific iPSCs from S. Yamanaka’s laboratory were infected by c-JUN lentivirus (MOI = 2) and then the expression of c-JUN and OCT4 were examined by western blot analysis as described in the Materials and Methods. * non-specific band. (D): Invasion activities of mouse hepatocyte-specific iPSCs infected with c-JUN (mHep-iPS + c-JUN) and control lentivirus (nHep-iPS) were examined as described in the Materials and Methods. Data are represented as the mean (n = 3) ± SEM (**, p < .01).
Taken together, our data support the hypothesis that rG2-DC-1C cells acquire “cancer stem cell”-like characteristics via the enhancement of the expression of OCT4 and c-JUN. Moreover, elevated expression of OCT4 and c-JUN was observed in specimens taken from patients with liver cancer (Fig. 6 and Table 1), and their expression was significantly intercorrelated (Table 1). The present clinical study has several implications; first, the coexpression of OCT4 and c-JUN in HCC is consistent with the in vitro data pertaining to OCT4/c-JUN positive feedback regulation (Fig. 6D). Second, OCT4 expression was detected in small tumors, which suggests that OCT4 plays a critical role in tumor initiation. Third, the absence of correlation between hepatitis virus infection and OCT4 expression suggests that both risk factors contribute to HCC development via different pathways. Approximately 40% of combined overexpression of OCT4 and c-JUN in primary hepatocytes via infection with recombinant viruses yielded tumor formation after xenotransplantation, and had dramatically reduced
survival rates. These results imply that identification of other critical OCT4 targets may be necessary to elucidate comprehensively the mechanism underlying the OCT4-initiated HCC development.

**CONCLUSION**

The present study demonstrates that OCT4 induced CSC-like properties and enhanced invasion capacity, thus contributing to the tumorigenesis and invasiveness of rG2-DC-1C cells via the activation of c-JUN. Therefore, the transactivation of c-JUN by OCT4 in the nucleus via the OCT4-binding elements located in the promoter of c-JUN seems to be critical for the induction of CSC-like characteristics. We propose that OCT4 and c-JUN may be biomarkers and therapeutic targets in liver cancers.

**ACKNOWLEDGMENTS**

We thank H.C. Huang and H.Y. Tseng of Welgene Biotech for RNA-sequencing works and Drs. C-H., Yen, Y-M., Chen, M. Noguchi, K. Kato, and K. Nagata for acquisition of materials, data and discussion. Recombinant lentiviruses including CSIV-CMV-MCS-IRE52-Venus-encoded human OCT4, KLF4, SOX2, C-MYC (OSKM), shTP53, CSII-EF-MCS-IRE52-Venus, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev were provided by RIKEN BRC which is participating in the National Bio-Resources Project of the MEXT, Japan. This work was supported by the grants from Ministry of Science and Technology (MOST-104-2320-B-037-033-My2, MOST-104-2314-B-037-002, MOST-104-2314-B-016, MOST-104-2314-B-037-043), National Health Research Institutes (NHRI-Ex104-10416SI), and Kaohsiung Medical University (KMU-TP104A04, KMU-TP104G00, 01, 03, 04, KMU-TP104E24, KMU-DT104001).

**AUTHOR CONTRIBUTIONS**

K.K.K, L.C.S, and Y.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript; L.K.T.: collection and/or assembly of data, provision of study material or patients; Y.Y.H., T.M.H., W.K., and K.C.C.: collection and/or assembly of data, administrative support; L.Y.C. and L.Y.L.: data analysis and interpretation; M.H., N.Y., and S.S.: collection and/or assembly of data, data analysis and interpretation; R.E.: manuscript writing; L.C.L., W.C.C., C.Y.C., and W.D.C.: provision of study material or patients.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.

**REFERENCES**

1. Tschochatzis EA, Meyer T Burroughs AK. Hepatocellular carcinoma. N Engl J Med 2012;366:92–93.
2. Bruix J, Boix L, Sala M et al. Focus on hepatocellular carcinoma. Cancer Cell 2004;5:215–219.
3. Eaves CJ. Cancer stem cells: Here, there, everywhere? Nature 2008;456:581–582.
4. Lowry WE, Richter L, Yachecchio R et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci USA 2008;105:2883–2888.
5. Park IH, Zhao R, West JA et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 2008;451:141–146.
6. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–676.
7. Wernig M, Messner A, Foreman R et al. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 2007;448:318–324.
8. Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.
gene regulation in pluripotent stem cells: of octamer-4 in serous and mucinous ovarian cancer progression in hepatocellular carcinoma stem cell phenotypes through Oct4-cancer and their association with distant cancer recurrence after chemotherapy. Ann Surg Oncol 2009;16:3488–3498.

10. Hu T, Liu S, Breiter DR et al. Octamer 4 small interfering RNA results in cancer stem cell-like cell apoptosis. Cancer Res 2008;68: 6533–6540.

11. Lopez-Terrada D, Cheung SW, Finegold MI et al. Hep G2 is a hepatoblastoma-derived cell line. Hum Pathol 2009;40:1512–1515.

12. Wang SW, Wang SS, Wu DC et al. Androgen receptor-mediated apoptosis in bovine testicular induced pluripotent stem cells in response to phthalate esters. Cell Death Dis 2013;4:e907.

13. Pan J, Nakade K, Huang YC et al. Suppression of cell-cycle progression by jun dimerization protein-2 (JDP2) involves down-regulation of cyclin-A2. Oncogene 2010;29: 6245–6256.

14. Tanigawa S, Lee CH, Lin CS et al. Jun dimerization protein 2 is a critical component of the Hri2/MafK complex regulating the response to ROS homeostasis. Cell Death Dis 2013;4:e921.

15. Liu C, Kelnar K, Liu B et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 2011;17:211–215.

16. Yoon CH, Kim MJ, Kim RK et al. c-jun N-terminal kinase has a pivotal role in the maintenance of self-renewal and tumorigenicity in glioma stem-like cells. Oncogene 2012;31:4655–4666.

17. Ibrahim EE, Babaee-Jadidi R, Saadeddin A et al. Embryonic NANOG activity defines colorectal cancer stem cells and modulates through AP1- and TCF-dependent mechanisms. Stem Cells (Dayton, Ohio) 2012;30: 2076–2087.

18. Apostolou P, Toloudi M, Ioannou E et al. AP-1 gene expression levels may be correlated with changes in gene expression of some stemness factors in colon Carcinomas. J Signal Transduct 2013;2013:497383.

19. Chang CC, Shieh GS, Wu P et al. Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. Cancer Res 2008;68:6291–6291.

20. Wang XQ, Ongkeko WM, Chen L et al. Octamer 4 (Oct4) mediates chemotherapeutic drug resistance in liver cancer cells through a potential Oct4-AKT-ATF-binding cassette G2 pathway. Hepatology 2010;52: 528–539.

21. Bannister AJ, Kouzarides T. CBP-induced stimulation of c-fos activity is abrogated by E1A. EMBO J 1995;14:4758–4762.

22. Katryar S, Jiao X, Wagner E et al. Somatic excision demonstrates that c-jun induces cellular migration and invasion through induction of stem cell factor. Mol Cell Biol 2007;27:1356–1369.

23. Jiao X, Katryar S, Willmarth NE et al. c-jun induces mammary epithelial cellular invasion and breast cancer stem cell expansion. J Biol Chem 2010;285:8218–8226.