Cyclin G1 Expands Liver Tumor-Initiating Cells by Sox2 Induction via Akt/mTOR Signaling

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Abstract

Recurrence and chemoresistance of liver cancer has been attributed to the existence of liver tumor-initiating cells (T-ICs). It is important to decipher the molecular mechanism for acquisition of drug resistance and to design combinatorial therapeutic strategies. Cyclin G1 has been shown to play a pivotal role in initiation and metastasis of hepatocellular carcinoma. In this study, we found that enhanced cyclin G1 expression was associated with drug resistance of hepatoma cells and higher recurrence rate in hepatocellular carcinoma patients. Expression of cyclin G1 was elevated in liver T-ICs and closely correlated with the expression of liver T-IC markers. Forced cyclin G1 expression remarkably enhanced self-renewal and tumorigenicity of hepatoma cells. Cyclin G1 overexpression dramatically upregulated the expression of Sox2 both in vitro and in vivo, which was impaired by chemical inhibitors of Akt/mTOR signaling. Furthermore, blockade of Akt/mTOR signaling or interference of Sox2 expression suppressed cyclin G1–enhanced self-renewal, chemoresistance, and tumorigenicity of hepatoma cells, indicating that cyclin G1 expands liver T-ICs through Sox2 induction via Akt/mTOR signaling pathway. These results suggest that cyclin G1–induced liver T-IC expansion contributes to the recurrence and chemoresistance of hepatoma, and cyclin G1 may be a promising biomarker for individualized therapy of hepatocellular carcinoma patients.

Introduction

Hepatocellular carcinoma is the fifth most common cancer in the world and the second leading cause of cancer death in men (1). Despite the improvement of resection and liver transplantation, long-term survival of hepatocellular carcinoma patients remains unsatisfactory due to the frequent recurrence after hepatectomy (2). It has been reported that 5-year recurrence rate of liver cancer exceeds 70% to date (3). Moreover, the majority of hepatocellular carcinoma patients was diagnosed at advanced stages and not eligible for surgical treatment. Unfortunately, chemoresistance nature of hepatocellular carcinoma makes it difficult to eradicate cancer cells by means of either conventional chemotherapy or transarterial chemembolization (4). It is thereby urgent to elucidate the molecular pathogenesis of hepatocellular carcinoma recurrence and chemotherapy, so that novel strategy of treatment can be developed.

Most recent findings strongly support the concept that tumors are generated and maintained by a small-defined subset of undifferentiated cells which are termed “cancer stem cells” or “tumor-initiating cells” (T-ICs; refs. 5–9). These cells are able to self-renew and differentiate into the bulk tumor population (10). Being a built-in population of tumor, these T-ICs can survive chemotherapy and repopulate the tumor (11). Accumulating evidence has showed that T-ICs exist in various tumors, including leukemia (12), glioma (13), breast (14), and colon cancer (15). Liver T-ICs have also been identified by several cell surface antigens such as CD133 (16), CD90 (17), CD24 (11), and epithelial cell adhesion molecule (18). Existence of T-ICs in hepatocellular carcinoma is likely to be one of the most principle reasons why current oncologic therapies exhibit poor effectiveness (10, 19, 20). Eradication of liver T-ICs should be essential to achieve stable, long-lasting remission, and even a cure of hepatocellular carcinoma. However, specific therapy targeting T-ICs has yet to be developed due to lack of knowledge on the regulation of the small reservoir for tumor cells. It is thereby urgent to elucidate the molecular mechanism of T-IC expansion so that novel strategy for hepatocellular carcinoma treatment can be achieved.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Cyclin G1 was initially discovered as a novel member of cyclin family with homology to c-src (21). Importantly, cyclin G1 is transcriptionally activated by p53, and in turn negatively regulates p53 family proteins (22). In experimental hepatocarcinogenesis, loss of cyclin G1 is associated with a significantly lower tumor incidence after carcinogenic challenge (23). Interaction between cyclin G1 and hepatocyte-specific miR-122a might abrogate p53-mediated inhibition of HBV replication and therefore contribute to viral persistence and carcinogenesis (24). Our previous data also suggest that cyclin G1 promotes epithelial–mesenchymal transition of hepatoma cells and facilitates hepatocellular carcinoma metastasis (25). However, role of cyclin G1 in chemoresistance and hepatocellular carcinoma recurrence remains largely unknown. In this study, we identified the novel function of cyclin G1 in liver T-IC expansion and clarified its clinical significance in hepatocellular carcinoma recurrence and chemotherapy.

Materials and Methods

Patients and liver samples

One hundred and thirty-eight hepatocellular carcinomas were randomly retrieved from hepatocellular carcinoma patients receiving curative resection in Eastern Hepatobiliary Surgery Hospital, Shanghai, China, from September 2001 to July 2007 (see detailed clinicopathologic features in Supplementary Table S1). All patients were followed up until March 2010, with a median observation time of 42 months. Matched pairs of primary hepatocellular carcinoma samples and adjacent liver tissues were used for the construction of a tissue microarray (in collaboration with Shanghai Biochip Company Ltd., Shanghai, China). Immunohistochemistry (IHC) was conducted on tissue microarray slides. Eighty-seven pairs of fresh human hepatocellular carcinoma with peri-cancerous tissues diagnosed by pathologist were obtained from Eastern Hepatobiliary Surgery Hospital. Patient samples were obtained following informed consent according to an established protocol approved by the Ethic Committee of Eastern Hepatobiliary Surgery Hospital.

Cell lines and recombinant virus

SMMC-7721 and HepG2 were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences where they were characterized by cell vitality detection, DNA fingerprinting, isozyme detection, and mycoplasma detection. These cell lines were immediately expanded and frozen so that they could be restarted every 3 to 4 months from a frozen vial of the same batch of cells. The cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen Corporation) supplemented with 10% FBS (Gibco, Invitrogen). SMMC-7721 or HepG2 were infected by Lenti-virus expressing GFP or cyclin G1, and stable cell lines were established as described previously. Lenti-virus expressing cyclin G1 or GFP was generated using Lenti-X Expression System (Clontech Laboratories, Inc.). Adenovirus encoding cyclin G1 (Ad-cyclin G1) and green fluorescent protein (Ad-GFP) were generated using Ad-Max Adenovirus Vector (Microbix, Inc.).

Luciferase reporter assays

SMMC-7721 cells were transfected with pGL3-Sox2p-enhancer-Luc (kindly provided by Prof. A. García Martín, Spain) using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured in duplicate by Synergy 2 Multi-detection Microplate Reader (BioTek Instruments, Inc.).

Real-time PCR

Quantitative PCR was conducted using SYBR Green PCR Kit (Applied Biosystems) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA level of specific genes was normalized against β-actin. The sequences of primers used in this study were listed in Supplementary Table S2.

Western blot

The hepatocellular carcinoma cell extract was separated on polyacrylamide-SDS gels, transferred, and probed with a specific primary antibody. The protein band, specifically bound to the primary antibody, was detected using an IRDye 800CW-conjugated secondary antibody and LI-COR imaging system (LI-COR Biosciences). The manufacturer information of primary antibodies was provided in Supplementary Table S3.

Immunohistochemistry and tissue microarray analysis

IHC of tumor sections or tissue microarray was conducted using the antibodies listed in Supplementary Table S3. Briefly, the sections were incubated with primary antibody at 4°C overnight and then with horseradish peroxidase-conjugated secondary antibody at 37°C for 30 minutes. The sections were finally incubated with diaminobenzidine and counterstained with hematoxylin for detection. Assessment of the staining was based on the percentage of positively stained cells and the staining intensity using software Image-Pro Plus 6.0 (Media Cybernetics, Inc.). Integrated optical density of all the positive staining in each photograph was measured, and its ratio to total area of each photograph was calculated as density. High expression of cyclin G1 is referred to as the signal of positive staining was higher than the median value.

Immunofluorescence staining

Frozen sections of fresh human hepatocellular carcinoma tissues were incubated with anti-CD133, anti-cyclin G1, and anti-Sox2 primary antibody, followed by fluorescent staining with Alexa Fluor 488-conjugated immunoglobulin G (IgG) or Alexa Fluor 555-conjugated IgG (Invitrogen). Nuclear staining was conducted by Hoechst 33342 in tissue samples. Representative images were captured with Olympus IX70.
Side population discrimination assay

Once the cells had reached a logarithmic growth phase, they were analyzed by fluorescence-activated cell sorting (FACS). Cells were digested with 0.25% trypsin (Sigma-Aldrich), washed twice with calcium/magnesium-free PBS, resuspended in ice-cold DMEM culture (supplemented with 2% FBS) at a concentration of $1 \times 10^6$ cells/mL, and incubated at $37^\circ C$ in a 5% CO$_2$ incubator for 10 minutes. The DNA binding dye, Hoechst 33342 (Sigma-Aldrich), was then added to a final concentration of 5 µg/mL and the cells were incubated for 90 minutes in the dark with interval mixing. The cells were washed twice with PBS before 1 µg/mL propidium iodide (Sigma-Aldrich) was added, and the cells were kept at 4°C in the dark prior to FACS analysis using the EPICS XL flow cytometer (Beckman Coulter). Because Hoechst 33342 extrudes from cells using verapamil (a calcium ion tunnel antagonist)-sensitive ABC transporters, a subset of the cells were incubated with 50 µmol/L verapamil for 30 minutes at $37^\circ C$ before Hoechst 33342 to determine whether this would block the fluorescent efflux of SP cells.

Apoptosis assay

Sorafenib-induced cell apoptosis was determined using the Vybrant Apoptosis Kit according to the manufacturer’s instructions (Molecular Probes) and were immediately analyzed by the EPICS XL flow cytometer (Beckman Coulter). Because Hoechst 33342 extrudes from cells using verapamil (a calcium ion tunnel antagonist)-sensitive ABC transporters, a subset of the cells were incubated with 50 µmol/L verapamil for 30 minutes at $37^\circ C$ before Hoechst 33342 to determine whether this would block the fluorescent efflux of SP cells.

Spheroid assay

Spheroid assay was conducted as previously described (26). For fresh clinical tissue specimens, single-cell suspensions of $1 \times 10^4$ primary hepatocellular carcinoma cells were seeded in 6-well ultra-low attachment microplates.

In vitro limiting dilution assay

SMCC-7721 cyclin G1 or their control cells were seeded into 96-well ultra-low attachment culture dishes at various cell numbers and incubated under spheroid condition for 7 days. Colony formation was assessed by visual inspection. Based on the frequency of wells without colony, proportion of T-ICs was determined using Poisson distribution statistics and the L-Calc Version 1.1 software program (Stem Cell Technologies, Inc.).

Chemoresistant tumor model

Subcutaneous xenografts were established with the SMMC-7721 hepatocellular carcinoma cell line. The animals used to test the treatment were 4- to 6-week-old male athymic nude mice (BALB/c-nu/nu). Treatment was started once the size of the xenograft reached approximately 4 mm in diameter. The mice were randomly assigned into 2 groups, each consisting of 6 mice. They were given cisplatin (2 mg/kg) or PBS intraperitoneally every day for 1 week.

Xenograft formation

SMCC-7721 cyclin G1 or the control cells were mixed with matrigel at a ratio of 1:1 and then injected subcutaneously into NOD-SCID mice at variant cells per mouse. Mice were sacrificed after 5 weeks postinoculation and tumors samples were measured and collected. Rapamycin and Ly294002 were administered intraperitoneally at the dose of 1.5 mg/kg daily or 25 mg/kg 3 times a week, respectively. The mice were monitored twice weekly for palpable tumor formation and euthanized 4 or 6 weeks after transplantation to assess tumor formation. Tumors were measured using a Vernier caliper, weighed, and photographed. A portion of the subcutaneously tumor tissue was collected, fixed in 10% formaldehyde, and embedded in paraffin for tumor pathology analysis.

Statistical analysis in this study was calculated with SPSS 14.0 (SPSS Inc.). Data were expressed as “mean value ± SEM.” The significance of mean values between 2 groups was analyzed by Student t test. All differences were two-sided. multivariate analyses were based on the Cox proportional hazards regression model. Kaplan-Meier and log-rank analysis was used to assess the patient survival between subgroups. A $P$ value less than 0.05 was considered statistically significant.

Results

Cyclin G1 expression is associated with chemoresistance and recurrence of hepatocellular carcinoma

Cisplatin resistant hepatocellular carcinoma xenograft tumors were established in immunocompromised mice mimicking the clinical situation in which the patients underwent chemotherapy (11). As shown in Fig. 1A, expression of cyclin G1 was notably increased in the T-IC enriched residual chemoresistant tumors as compared with the control tumors. Cisplatin-resistant SMMC-7721 cells were generated by cisplatin (0.25 µg/mL) treatment for 4 weeks in vitro. Consistently, cisplatin-resistant hepatocellular carcinoma cells exhibited a significant increased expression of cyclin G1 compared to control hepatocellular carcinoma cells (Supplementary Fig. S1A). Sorafenib is currently the only systemic targeted drug-displaying efficacy in advanced stages of hepatocellular carcinoma. As shown in Fig. 1B, cyclin G1 expressing hepatoma cells were more resistant to sorafenib treatment than control cells. Moreover, disease-free survival analysis revealed that hepatocellular carcinoma patients with high cyclin G1 expression possess higher hepatocellular carcinoma recurrence rate than those with low cyclin G1 levels (Fig. 1C). In multivariate analysis, either vascular invasion ($P < 0.05$), tumor size (>5 cm in diameter, $P < 0.05$), or cyclin G1 overexpression ($P < 0.05$) was independent prognostic factor for hepatocellular carcinoma recurrence (Fig. 1D). Clinical correlation study showed that overexpression of cyclin G1 is associated with the increased vascular invasion of hepatocellular carcinoma ($P < 0.05$).
(Supplementary Table S4). The patients with elevated cyclin G1 expression and vascular invasion displayed even worse prognosis (Supplementary Fig. S1B).

**Cyclin G1 levels are correlated with liver T-IC characteristics**

Existence of T-ICs is considered as the principle factor of chemoresistance and cancer recurrence. To elucidate whether cyclin G1 was involved in regulation of T-ICs, spheroid assay was conducted using primary human hepatoma cells. As shown in Fig. 2A, expression of cyclin G1 was significantly elevated in the spheroids compared with monolayer primary cultured cells. CD133⁺ or OV6⁺ liver T-ICs sorted from trypsinized spheroids of hepatoma cells displayed higher level of cyclin G1 compared with the counterparts (Fig. 2B). Significant correlation between expression of cyclin G1 and CD133 or CD90, which are considered as potential markers of liver T-ICs, was observed in spheroids derived from freshly isolated human hepatocellular carcinoma cells (Fig. 2C). In addition, CD133 expression was also detected in cyclin G1 overexpressing hepatoma cells from fresh frozen sections by fluorescent IHC assay (Fig. 2D).

**Cyclin G1 promotes the expansion of liver T-ICs in hepatoma cells**

To explore the role of cyclin G1 in liver T-IC regulation, adenovirus-mediated cyclin G1 introduction and cyclin G1 stable transfectant were analyzed. Overexpression of cyclin G1 remarkably upregulated the expression of CD133 and CD90 in both stable transfectant and adenovirus-infected hepatoma cells (Fig. 3A). Flow cytometry analysis detected the increased percentage of CD133 positive cells induced by cyclin G1 overexpression in hepatoma cells (Supplementary Fig. S1C and S1D). Enforced cyclin G1 expression notably facilitated the spheroid formation of hepatocellular carcinoma cell lines and primary hepatoma cells from patients (Fig. 3B and
Supplementary Fig. S2A and B). Consistently, hepatoma cells with cyclin G1 overexpressing exhibited a significant increased proportion of side population cells, which was considered as hepatic T-ICs, compared to their counterpart (Supplementary Fig. S2C). In vitro limiting dilution assay revealed that overexpression of cyclin G1 significantly increased the proportion of T-ICs in hepatoma cells (Fig. 3C). Moreover, hepatoma cells expressing cyclin G1 exhibit enhanced tumor initiating capacity (27) in NOD-SCID mice compared with the control cells (Table 1 and Fig. 3D).

**Figure 2.** Cyclin G1 levels are correlated with liver T-IC characteristics. A, primary culture of hepatocellular carcinoma cells from fresh hepatocellular carcinoma clinical samples was conducted in monolayer or ultra-low attachment condition. Representative picture is shown. The expression of cyclin G1 was analyzed by real-time PCR and compared with CD133 or OV6 hepatocellular carcinoma cells sorted from trypsinized spheroid was analyzed by quantitative real-time PCR and compared with CD133 or OV6 counterparts. Error bars, SEM. \* P < 0.05. B, expression level of cyclin G1 in CD133 or OV6, cyclin G1–induced T-IC expansion, expression of key stemness regulatory genes was determined. Transcription of Sox2

**Figure 3.** Cyclin G1 promotes the expansion of liver T-ICs in hepatoma cells. A, expression of CD133 and CD90 was analyzed in stable cell line or adenovirus-infected hepatoma cells as indicated. B, primary human hepatocellular carcinoma cells were infected by adenovirus carrying cyclin G1 or GFP, and spheroid formation assay was conducted (n = 5). Representative picture is shown. Error bars, SEM. \* P < 0.05 (for diameters of spheroid, left) and between cyclin G1 and CD90 expression (n = 30; r = 0.796; P < 0.05; right) with a scatter plot. C, colocalization of cyclin G1 and CD133 was observed in fresh frozen sections using fluorescent IHC assay. Nucleus is stained by Hoechst 33342 (blue; n = 5). Representative picture is shown.

**Sox2 is involved in cyclin G1–enhanced T-IC expansion**

To disclose the molecular mechanism underling cyclin G1–induced T-IC expansion, expression of key stemness regulatory genes was determined. Transcription of Sox2
was significantly upregulated in cyclin G1–expressing hepatoma cells (Fig. 4A). Close correlation between cyclin G1 and Sox2 was observed in 87 hepatocellular carcinoma tissues of patients (Supplementary Fig. S3A–S3C). Intriguingly, the correlation was more significant in primary cultured spheroids from hepatoma cells of patients (Fig. 4B). Consistently, colocalization of cyclin G1 and Sox2 was also observed in clinical samples of hepatocellular carcinoma patients (Supplementary Fig. S3D). Sox2 promoter reporter assay showed that cyclin G1 overexpression remarkably activated Sox2 expression and introduction of shRNA targeting cyclin G1 robustly reduced Sox2 promoter activity (Supplementary Fig. S4A). Induction of Sox2 by cyclin G1 was also confirmed in the subcutaneous xenografts of hepatoma cells with cyclin G1 overexpression (Fig. 4C and Supplementary Fig. S4B).

Considering Sox2 upregulation was reported to affect cyclin D1 expression, we determined the cyclin G1 level in hepatoma cells with Sox2 overexpression (28). As shown in Supplementary Fig. S4D, overexpression of Sox2 notably promoted the spheroid formation of hepatoma cells whereas cyclin G1 expression was not influenced. Unsurprisingly, knockdown of Sox2 by its specific siRNA remarkably abolished cyclin G1 promoted self-renewal (Fig. 4D and Supplementary Fig. S4C).

### Cyclin G1 upregulates Sox2 expression via Akt/mTOR signaling

Akt/mTOR signaling is rapidly emerging as critical signaling for the regulation of stemness-related genes and T-IC maintenance (29). It was reported that inhibition of mTOR by rapamycin could lead to activation of Akt, resulting from abrogating the feedback inhibition mediated by activated mTOR pathway (30). To completely block Akt/mTOR signaling, we treated the cells with Rapamycin and Ly294002 in combination (Supplementary Fig. S5A) following previous studies (31, 32). As shown in Fig. 5A and Supplementary Fig. S5B, cyclin G1–elicited activation of canonical mTOR targets and induction of Sox2 were dramatically eliminated in hepatoma cells exposed to Akt/mTOR inhibitors. Consistently, suppression of Akt/mTOR signaling by either chemical inhibitors or small interference RNA evidently attenuated cyclin G1–enhanced spheroid formation of hepatoma cells (Fig. 5B and Supplementary Fig. S5C). Sox2 overexpression remarkably enhanced spheroid formation of cyclin G1

<table>
<thead>
<tr>
<th>Stable transfectants</th>
<th>Tumor formation</th>
<th>T-ICs frequency</th>
<th>P value</th>
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<tr>
<td>Cell number</td>
<td></td>
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<td></td>
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<tr>
<td>10³</td>
<td>10⁴</td>
<td>3 × 10⁴</td>
<td>10⁵</td>
</tr>
<tr>
<td>SMMC-7721 GFP</td>
<td>0/4</td>
<td>1/6</td>
<td>2/4</td>
</tr>
<tr>
<td>SMMC-7721 Cyclin G1</td>
<td>1/4</td>
<td>4/6</td>
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Table 1. Frequency of T-ICs in stable transfectants of SMMC-7721 cell populations

![Figure 4. Sox2 is involved in cyclin G1–enhanced T-IC expansion. A, transcription of Oct4 (POU5F1), Nanog, Bmi1, c-Myc, Klf4, and Sox2 in SMMC-7721/cyclin G1 or SMMC-7721/GFP cells was analyzed by real-time PCR. B, significantly positive correlations were found between cyclin G1 and CD133 mRNA expression in spheroids derived from primary isolated human hepatocellular carcinoma cells. C, expression of Sox2 in tissue samples derived from xenografts in NOD-SCID mice was determined by Western blot. D, SMMC-7721/cyclin G1 or SMMC-7721/GFP cells were transfected with siRNA-targeting Sox2, and spheroid formation assay was conducted. Error bars, SEM. ✪, P < 0.05.](http://mct.aacrjournals.org/doi/10.1158/1535-7163.MCT-13-0099)
stable transfectant and the control cells at the presence of Akt/mTOR inhibitors (Supplementary Fig. S5D). Cyclin G1–mediated Sorafenib resistance was also abolished (Fig. 5C). Moreover, enhanced tumor initiating capacity of cyclin G1–expressing hepatoma cells in NOD-SCID mice was significantly diminished at the presence of Akt/mTOR signaling inhibitors (Fig. 5D). Collectively, these data showed that cyclin G1–induced T-IC expansion is via, at least partially, Akt/mTOR/Sox2 pathway.

Discussion

It is traditionally believed that accumulation of genetic and epigenetic mutations in regenerating mature hepatocytes during chronic liver injury leads to hepatocellular carcinoma occurrence (33). However, more and more evidence favors the hypothesis of liver T-ICs, which occupy a rare subpopulation within hepatocellular carcinoma, are responsible for hepatocellular carcinoma initiation and progression (34, 35). Numerous studies have evidenced the existence of liver T-ICs which display distinct surface marker pattern, and have indicated their significance in prognosis and treatment of the patients. However, no therapy regimen targeting liver T-ICs has been developed yet due to the lack of specific molecular targets. In this study, we elucidated the pivotal role of cyclin G1 in liver T-IC expansion with clinical relevance and disclosed the underlying molecular mechanism.

It has been widely accepted that the hepatocellular carcinoma development is attributed to the propagation of liver T-ICs, which are capable of self-renewal and are responsible for tumor initiation and chemoresistance. However, the molecular events underlying liver T-IC maintenance and expansion remains largely unclear. Considering enrichment of T-IC populations could be achieved upon chemotherapy because of their unique survival mechanism, we enriched liver T-ICs by establishing chemoresistant hepatocellular carcinoma xenograft tumors mimicking the clinical chemotherapy
increased the proportion of CD133+ cells has been accepted as distinct T-IC marker for hepatoma cells have been verified to possess T-IC characteristics and CD133 has been accepted as distinct T-IC marker for hepatocellular carcinoma (18, 19). Intriguingly, our data showed that forced cyclin G1 expression significantly increased the proportion of CD133+ hepatoma cells suggesting the regulatory role of cyclin G1 in liver T-ICs expansion. In further study, we found that cyclin G1 overexpression not only increased the proportion of liver T-ICs in hepatoma cells, but also enhanced self-renewal and tumor initiation capacity of hepatoma cells. These findings implicate that cyclin G1 is able to promote the expansion of liver T-ICs.

In 2006, Shinya Yamanaka and his team reported that expression of only 4 genes (SOX2, Oct-4, c-Myc, and Klf-4) were necessary to reprogram mouse fibroblasts into pluripotent stem cells. The polycomb gene product Bmi1 and stemness gene Nanog were also illustrated to regulate the self-renewal and contribute to the expansion of liver T-ICs (11, 36). These stemness-related genes are conspicuously downregulated in the differentiated somatic cells. In this study, we observed the prominent induction of Sox2 but not other stemness-related genes in hepatoma cells with cyclin G1 overexpression. In addition, we found a significant correlation between the levels of cyclin G1 and Sox2 in a set of hepatocellular carcinoma tissues and primary cultured spheroids from hepatocellular carcinoma tissues of patients. Overexpression of cyclin G1 significantly upregulated Sox2 expression in both cultured hepatoma cells and xenograft tumors. These data suggested a crosstalk between cyclin G1–activated signaling and Sox2 induction.

mTOR is rapidly emerging as a critical hub molecule required for cell survival and proliferation (37, 38). Recent studies have also linked mTOR activity to T-IC maintenance and expansion (39). We previously reported that cyclin G1 could activate Akt through interaction with p85, the regulatory subunit of PI3-K, which leads to EMT and metastasis of hepatoma cells (25). It is interesting to examine whether cyclin G1–enhanced T-IC expansion is dependant on Akt/mTOR signaling. Consistent with previous studies, inhibition of mTOR by Rapamycin led to hyper-phosphorylation of Akt, suggesting the regulatory role of cyclin G1 in liver T-IC expansion.

In our study, we observed the prominent induction of Sox2 but not other stemness-related genes in hepatoma cells with cyclin G1 overexpression. In addition, we found a significant correlation between the levels of cyclin G1 and Sox2 in a set of hepatocellular carcinoma tissues and primary cultured spheroids from hepatocellular carcinoma tissues of patients. Overexpression of cyclin G1 significantly upregulated Sox2 expression in both cultured hepatoma cells and xenograft tumors. These data suggested a crosstalk between cyclin G1–activated signaling and Sox2 induction.

The majority of hepatocellular carcinoma cases are diagnosed at advanced stage and conventional chemotherapy or transarterial chemoembolization are largely inefficient due to its extreme chemoresistance. It is thereby important to design combinatorial therapeutic strategies to overcome the drug resistance of hepatocellular carcinoma. Sorafenib is currently the only targeted agent-displaying efficacy in advanced stages of hepatocellular carcinoma despite that the response rate of patients is comparatively low. In this study, we found that cyclin G1 overexpression facilitated the chemoresistance of hepatoma cells to Sorafenib suggesting the influence of cyclin G1 on hepatocellular carcinoma chemoresistance. Blockage of Akt/mTOR signaling not only diminished cyclin G1–mediated Sox2 induction, but also increased the sensitivity of hepatoma cells to Sorafenib, implicating the synergic effects of these agents. Hepatocellular carcinoma patients with higher cyclin G1 levels exhibit significant higher recurrence rate than those with lower cyclin G1 expression, indicating the significance of cyclin G1 in the prognosis of patients receiving surgical resection.

To our knowledge, this is the first report on the function of cyclin G1 in liver T-IC expansion with clinical relevance. Elucidating the essential role of cyclin G1 in Sorafenib resistance and recurrence of hepatocellular carcinoma might provide new insight into individualized therapy of hepatocellular carcinoma patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: W. Wen, G.-S. Feng, M.-C. Wu, J. Ding, H.-Y. Wang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Wen, C. Chen, L. Huang, S.-Z. Chen, D.-M. Xiang, L. Tang, D. Cao, H.-Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biosististics, computational analysis): W. Wen, T. Han, L. Huang, S.-Z. Chen, L. Tang, G.-S. Feng, H.-Y. Wang
Writing, review, and/or revision of the manuscript (i.e., reviewing other’s work for important intellectual content): W. Wen, T. Han, S.-Z. Chen, G.-S. Feng, M.-C. Wu, J. Ding, H.-Y. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Wen, S.-Z. Chen, W. Sun, L. Tang, M.-C. Wu, H.-Y. Wang
Study supervision: W. Wen, H.-Y. Wang

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