MYC Activates Stem-like Cell Potential in Hepatocarcinoma by a p53-Dependent Mechanism

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Abstract

Activation of c-MYC is an oncogenic hallmark of many cancers, including liver cancer, and is associated with a variety of adverse prognostic characteristics. Despite a causative role during malignant transformation and progression in hepatocarcinogenesis, consequences of c-MYC activation for the biology of hepatic cancer stem cells (CSC) are undefined. Here, distinct levels of c-MYC overexpression were established by using two dose-dependent tetracycline-inducible systems in four hepatoma cell lines with different p53 mutational status. The CSCs were evaluated using side population (SP) approach as well as standard in vitro and in vivo assays. Functional repression of p53 was achieved by lentiviral shRNA transduction. The results show that c-MYC expression levels have a differential impact on liver CSC characteristics. At low levels, c-MYC activation led to increased proliferation and enhanced CSC properties including activation of reprogramming transcription factors and CSC marker expression (e.g., NANOG, OCT4, and EpCAM), expansion of SP, and acceleration of tumor growth upon subcutaneous transplantation into immunocompromised mice. However, when exceeding a threshold level, c-MYC induced a proapoptotic program and loss of CSC potential both in vitro and in vivo. Mechanistically, c-MYC–induced self-renewal capacity of liver cancer cells was exerted in a p53-dependent manner. Low c-MYC activation increased spheroid formation in p53-deficient tumor cells, whereas p53-dependent effects were blunted in the absence of c-MYC overexpression. Together, our results confirm the role of c-MYC as a master regulator during hepatocarcinogenesis and establish a new gatekeeper role for p53 in repressing c-MYC–induced CSC phenotype in liver cancer cells. Cancer Res; 74(20); 5903–13. ©2014 AACR.

Introduction

Over the last decade, compelling evidence emerged in support of a cancer stem cell (CSC) origin in many solid tumors including hepatic cancers. The CSCs are believed to encompass a rare subtype of highly tumorigenic cells that possess self-renewal capacity and promote malignant progression (1). Furthermore, because CSCs are thought to evade chemotherapy due to high endogenous expression of chemoresistance transporters, they are posited to be the cause of tumor relapse. For these reasons, CSCs are of great translational importance with a variety of therapeutic implications. Several recent studies prospectively isolated and evaluated putative hepatic CSCs by using surrogate characteristics such as xenograft tumor growth, expression of stem cell–associated antigens (e.g., EpCAM, CD133, and CD131), side population (SP) analysis, as well as spheroid formation capacity (2, 3). Despite some obvious limitations, these approaches improved our understanding of liver CSC biology, and allowed a reproducible characterization of the CSCs and exploration of the molecular drivers promoting CSC unique oncogenic properties.

c-MYC is one of the most commonly activated oncogenes implicated in the development of human cancers including liver cancer. c-MYC is involved in the regulation of 15% of genes in the human genome (4) associated with diverse biologic processes, such as cell growth, apoptosis, metabolism, and more recently microRNAs (5, 6). c-MYC regulates protumorigenic transcriptional factors such as KRAS, AKT, and PTEN (7), as well as p53, which plays a key role in c-MYC–induced apoptosis (8). Furthermore, c-MYC is also reported to be an essential regulator of self-renewal and pluripotency of embryonic stem (ES) cells as well as induced pluripotent stem cells (iPSC; refs. 9, 10).

The importance of c-MYC for liver cancer development is documented in several mouse models in which c-MYC overexpression efficiently and consistently drives liver cancer formation (11–13). We and others have established the contribution of c-MYC in the pathogenesis of human liver cancer (14–16) and demonstrated that induction of c-MYC is required for oncogenic reprogramming of terminally differentiated hepatocytes into hepatic CSC (17). Shachaf and colleagues...
(18, 19) showed that inactivation of c-MYC induced apoptosis and caused sustained regression of invasive liver cancer, confirming its essential role for the maintenance and progression of liver cancer. High c-MYC expression was found in SP cells from small-cell lung cancer (20) and tumor spheres formed by rhabdomyosarcoma cell lines (21). Recent reports also showed that MYC-centered regulatory networks activated in ES cells are enriched in some cancers, especially in the CSCs, conferring metastatic potential and poor outcome (22, 23). These results suggest that c-MYC can maintain a stem-like phenotype in pluripotent cells as well as in cancer cells. However, a direct relationship between c-MYC activation and hepatic CSCs is not yet established. In this study, we aimed to elucidate the significance of c-MYC for hepatic CSC biology by using controllable c-MYC expression systems. Our results establish a direct link between c-MYC expression levels and CSC properties. We further mechanistically demonstrate that c-MYC modulates the hepatic CSC phenotype in a p53-dependent manner.

Materials and Methods

Cell lines

PLC/PRF/5 (referred thereafter as PLC), Hep3B, and HepG2 were obtained from the American Type Culture Collection (ATCC), and Huh7 was obtained from Riken Cell Bank. PLC, Hep3B, and Huh7 were grown in DMEM and HepG2 in MEM supplemented with 2 mmol/L L-glutamine, 1 U/mL penicillin/streptomycin, and 10% FBS.

c-MYC Tet-On inducible system and transfection

Two vectors were used for expression of the reverse tetracycline-controlled transactivator protein (Tet-On). The pRetro-X-Tet-On Advanced retroviral vector was purchased from Clontech (catalog no. 632104), and retroviral particles were produced according to the manufacturer’s protocol. Cells transduced with the retroviral vector were selected by growth in G418 and used for immunocytochemistry. A lentiviral vector transduced with the retroviral vector were selected by growth produced according to the manufacturer’s protocol. Cells

Sphere-forming assay

Tumor cells (5 × 10^2 for PLC, Huh7, and HepG2 and 1 × 10^3 for Hep3B) were plated in ultra-low attachment plates (Costar) in serum-free growth medium containing 1% methylcellulose solution (R&D Systems) and B-27 (1 ×: Invitrogen), 25 ng/mL FGF, and 20 ng/mL EGFR in the presence or absence of doxycycline at varying concentrations. Doxycycline was added to medium every other day. The average sphere number and size were calculated using the NIH ImageJ software in three independent duplicate experiments. The spheres with diameter less than 50 μm were excluded from analysis. To assess self-renewal, the G1 spheres were dissociated and passaged every 7 days to produce G2–G5 spheres in the absence or presence of indicated doxycycline concentrations.

Tumor xenografts

All procedures were performed in accordance with the guidelines of the National Institutes of Health animal care committee. One hundred and 1,000 HepG2 cells cultured for 1 week in the presence or absence of varying concentrations of doxycycline were resuspended in 200-μL DMEM and Matrigel (BD Biosciences; 1:1) and injected into flanks of NOD/SCID mice (Frederick National Laboratory for Cancer Research, Frederick, MD). Doxycycline in concentrations of 10 and 100 ng/mL was added to drinking water containing 1% sucrose to reduce a bitter taste every 2 days. Tumor formation was monitored weekly by palpation for 8 weeks. Limiting dilution analysis was performed as described previously (http://bioinf. wehi.edu.au/software/elda/index.html; ref. 3).

SP analysis, RT-PCR, Western blotting, and immunofluorescence

Real-time PCR, Western blotting, immunofluorescence, and SP analysis were performed using standard assays described elsewhere (3). Primer sequences and antibodies are listed in Supplementary Tables S1 and S2. Fluorescently stained cells were viewed by a Zeiss LSM 510 NLO and Zeiss LSM 710 NLO (Zeiss).
Results

c-MYC is upregulated in SP and tumor spheres derived from liver cancer cells

c-MYC is overexpressed in CSC-enriched populations, such as SP from small-cell lung cancer (20), and tumor spheres formed by rhabdomyosarcoma cell lines (21). To address the functional importance of c-MYC for hepatic CSCs, we first took advantage of our recent microarray profiling of FACS-sorted SP and non-SP cells obtained from Huh7, WRL68, and KMCH liver tumor cell lines (3). Examination of microarray data identified MYC as one of the upregulated genes in the SP fractions as compared with the non-SP cells (Supplementary Fig. S1A). This was reinforced by the Gene Set Enrichment Analysis (GSEA) using "Myc Oncogenic Signature" (Supplementary Fig. S1B; ref. 28) demonstrating a significant enrichment of c-MYC–related genes in the SP fraction.

Next, we determined the c-MYC levels in sphere cultures established from three hepatoma cell lines including HepG2, Huh7, and PLC. We found that MYC expression increased progressively with sphere passaging in parallel with upregulation of pluripotency gene NANOG (Supplementary Fig. S1C; ref. 29). These data suggested a link between upregulation of MYC expression and enrichment of CSCs in liver cancer.

Conditional Tet operator–driven c-MYC expression

To address the functional role of c-MYC in the biology of liver CSCs, we examined the effects of overexpressing c-MYC in hepatoma cell lines. To minimize the concern related to c-
MYC–driven apoptosis (30), we used the doxycycline-inducible tetracycline (Tet)-On system, which allows precise control of c-MYC expression. Given recent findings that c-MYC can cooperate with p53 to promote acquisition of “stemness” properties and its role in driving the reprogramming of hepatic lineage cells into “CSCs” to form liver cancers (17), we have established and validated a Tet-On system in four liver cancer cell lines with different p53 status, including HepG2 with wild-type p53, Huh7, and PLC with mutant p53, and p53-null Hep3B (31).

We used two different Tet-On transactivator expression vectors, a lentiviral vector coexpressing EGFP and a retroviral vector with a neomycin-resistance marker, for cell biology assays and for immunofluorescence analysis, respectively. The Tet-On vectors were used in combination with a lentiviral vector expressing c-MYC and mCherry under the control of a tetracycline-responsive promoter (Fig. 1A).

Cells were transduced with both Tet-On and Tre-MYC particles followed by detailed analyses of doxycycline-dependent induction of c-MYC and reporter gene expression. Increasing doses of doxycycline (from 0 to 1 ng/mL) led to a marked dose-dependent upregulation of c-MYC both at mRNA (Fig. 1B) and protein (Fig. 1C) levels, including accumulation of phospho-MYC. Confocal microscopy confirmed that doxycycline-dependent mCherry expression was observed exclusively in c-MYC–overexpressing cells (Fig. 1D). In HepG2, Huh7, and PLC cells, the mCherry signal was first detected starting from 0.01 ng/mL of doxycycline and reached the...
maximum intensity at 1.0 ng/mL, referred hereafter as to “low” and “high” MYC activation doses, respectively (Supplementary Fig. S2A). In p53-null Hep3B cells, which were significantly more sensitive to c-MYC overexpression, the low and high c-MYC activation was achieved at 10-fold lower doxycycline concentrations, 0.001 and 0.1 ng/mL, respectively (Supplementary Fig. S2B). Consistent with published data (30), forced c-MYC expression in liver cancer cells caused dose-dependent changes in cell proliferation and apoptosis. Low levels of c-MYC activation in HepG2 cells caused a 2-fold increase in proliferation without affecting the rate of apoptosis as measured by the frequency of Ki67+ and TUNEL+ cells (Fig. 2A–D). This was paralleled by upregulation of cell-cycle (CDK2, cyclin D1, and CDC2) and antiapoptotic (BCL-2 and BCL-XL) proteins (Fig. 2E). A further increase in c-MYC expression (1 ng/mL of doxycycline) did not change the rate of cell proliferation (Fig. 2C) but caused a 10-fold increase in apoptosis (Fig. 2D) and upregulation of the proapoptotic proteins p53, p21, p27, and cleaved caspase-3 (Fig. 2E). Consequently, low levels of c-MYC activation shifted the balance in the growth rate toward proliferation without increasing cell death, whereas at high levels of c-MYC expression, the majority of tumor cells died
from apoptosis (Fig. 2F). These results were reproduced in Huh7 (Fig. 2B and F), as well as PLC and Hep3B cells (not shown). Collectively, these data demonstrate that Tet-controlled c-MYC expression is a reliable experimental system to conditionally regulate c-MYC activity in liver cancer cells.

Low levels of c-MYC activation increase the frequency of liver CSCs

Our previous experiments revealed that CSC-enriched SP cells expressed higher levels of MYC (Supplementary Fig. S1A). Now, we asked whether forced activation of c-MYC in liver cancer cells may affect the CSC properties. We have found that tetracycline-controlled c-MYC expression causes a dose-dependent increase in the size of the SP fraction in HepG2 (Fig. 3A), and Huh7 and PLC cell lines (Supplementary Fig. S3A). Low level of c-MYC activation was the most effective in HepG2 cells (40-fold increase in SP frequency), which possessed the smallest basal SP frequency (0.01%) as compared with Huh7 (4.5-fold increase) and PLC (4.1-fold increase). This was consistent with the upregulation of pluripotency and CSC-related proteins, such as NANOG, OCT4, BMI1, SOX2, and EpCAM, which peaked at the low levels of c-MYC activation as evidenced by Western blotting (Fig. 3B) and confocal microscopy (Fig. 3C and Supplementary Fig. S3B). A further increase in c-MYC activity was associated with a gradual reduction in the SP fraction (Fig. 3A and Supplementary Fig. S3A), and decreased expression of stem cell–associated proteins (Fig. 3B), suggesting that optimal levels of c-MYC activation contribute to the maintenance of CSCs in liver cancer. Similar data were obtained when we subjected HepG2, Huh7, Hep3B, and PLC cells to a tumor sphere assay (Fig. 3D and Supplementary Fig. S3C). A low increase in c-MYC activity strongly increased both the number (Fig. 3E) and the size (Fig. 3F) of primary spheres as compared with no activation groups. These differences were maintained throughout the sphere passaging for three to five generations (Fig. 4A–C and Supplementary Fig. S4A).
In contrast, high level of c-MYC activation failed to sustain a self-renewal potential of hepatic CSCs beyond the second or third sphere generation (Fig. 4 and Supplementary Fig. S4). We confirmed that c-MYC was activated during serial sphere passaging by confocal microscopy. Doxycycline-dependent activation of mCherry reporter gene was detected in the fifth sphere generation formed by HepG2 cells with tetracycline-controlled c-MYC expression (Fig. 4D).

To further substantiate the role of c-MYC in CSCs activation, we performed a series of c-MYC switch-on and switch-off experiments. To this aim, HepG2 and PLC cells were grown in nonadherent conditions both in the absence or presence of low doxycycline concentration (0.01 ng/mL) to achieve an optimal level of c-MYC activation. During the subsequent sphere passaging (P2–P5), the growth conditions either remained the same (I and IV) or switched to the presence (condition II) or absence (condition III) of doxycycline. B, kinetic changes in sphere frequency during c-MYC switch-on and switch-off activation. Data are mean ± SD determined in 12 wells for one of three independent experiments. Scale bar, 200 μm.

Next, we addressed the tumorigenic potential of liver cancer cells with different levels of c-MYC expression by using subcutaneous xenograft model. Limiting dilution analysis confirmed a higher frequency of tumor-initiating cells among the cells with low levels of c-MYC activation (Fig. 6A). In mice receiving low doxycycline concentration (10 ng/mL in drinking water) to activate the Tet operator-controlled c-MYC expression, as few as 100 HepG2 cells produced tumors in three of six injection sites as compared with the unstimulated cells (0 of 6) by 8 weeks after transplantation. The tumors in the "Low Myc activation" group also grew bigger (Fig. 6B–D). Morphologically, the tumors were typical hypervascular hepatocellular carcinoma without any apparent differences between groups (Fig. 6E). In mice receiving high doxycycline concentrations (100 ng/mL in drinking water), no tumors developed at 8 week after injecting 1,000 cells (Fig. 6A), most likely due to the cell loss by apoptosis. We confirmed that tumors continued to express mCherry, indicating that doxycycline supplementation in drinking water was sufficient for driving c-MYC activation (Fig. 6F). Thus, the low levels of c-MYC activation remarkably increased the pool of tumor-initiating cells.

The direct effect of c-MYC activation on long-term self-renewal ability of liver cancer cells in vitro.
The relationship between c-MYC–induced reprogramming and p53

It is noteworthy that the effects of c-MYC activation on CSC self-renewal varied between liver cancer cell lines. HepG2 cells with wild-type p53 maintained the long-term self-renewal albeit at different levels depending on c-MYC expression (Fig. 5B). The PLC cells with mutant p53 had a limited self-renewal, which was increased upon low levels of c-MYC activation (Supplementary Fig. S5B). In comparison, the p53-null Hep3B cells not only produced much fewer spheres than both HepG2 and PLC, but the Hep3B primary spheres lacked the ability to self-renew under the basal culture conditions (Supplementary Fig. S6A). However, very low levels of c-MYC activation in Hep3B cells caused a dramatic and steady increase both in the sphere-forming ability and self-renewal (Supplementary Fig. S6A). Consequently, Hep3B cells showed the biggest and HepG2 the smallest impact of low c-MYC activation on the long-term self-renewal potential, suggesting that c-MYC–driven CSC activation may be controlled by p53 (Supplementary Fig. S6B).

To address this hypothesis, we carried out loss-of-function experiments by generating a stable lentivirus-transduced HepG2 cell line expressing shRNAs against p53, and clonally isolating cells with effective p53 knockdown (Fig. 7A and B). Knockdown of p53 did not affect doxycycline-mediated c-MYC activation but significantly increased the levels of cyclin D1 expression (Fig. 7C) and growth rate (Supplementary Fig. S7), which was likely due to a reduced rate of apoptosis. Notably, in shp53-transduced HepG2 cells, enforced c-MYC expression caused a considerably stronger induction of CSC markers EpCAM, NANOG, and BMI1, as compared with the scramble shRNA-infected cells (Fig. 7C). Furthermore, knocking out p53 significantly enhanced both the sphere-forming capacity and long-term self-renewal in HepG2 cells upon low c-MYC activation (Fig. 7D and E). As a result, the ratio between the sphere numbers formed by shp53 HepG2 cells under condition of low and no c-MYC activation was steadily increasing (Supplementary Fig. S6C) similarly to that observed in Hep3B p53-null cells (Supplementary Fig. S6A). Together, the results suggest that p53 inactivation promotes c-MYC–induced reprogramming of liver cancer cells (Supplementary Fig. S6D).

Discussion

The importance of c-MYC for stem cells and CSCs is well recognized. c-MYC is one of the four key components required for the reprogramming of somatic cells into pluripotent stem cells (10), and effective induction of pluripotency is tightly regulated by several other factors including p53 (32). In this study, we used a Tet-inducible system that allowed a tight control of c-MYC expression in the context of hepatic CSCs. Our results provide evidence for
the direct role of c-MYC in maintaining CSC phenotype and suggest that c-MYC exerts its CSCs modulating activity in a p53-dependent manner.

In agreement with the previously published studies, we found that c-MYC exerted its actions in a dose-dependent manner (30, 33, 34). At low levels, induction of c-MYC increased oncogenic properties and induced proliferation in hepatoma cells via activation of CCND1 and CDK2, whereas upon exceeding a threshold level, which varied depending on liver tumor cell line, it led to upregulation of proapoptotic p53, p21, and p27 and caused cell death from apoptosis. We further show that depending on the expression levels, c-MYC can either repress or activate oncogenic memory in cancer/CSCs cells (18). Thus, c-MYC activation caused a dose-dependent upregulation of pluripotency and stemness genes NANOG, OCT4, BMI1, SOX2, and EpCAM, and a corresponding increase in the sphere-forming capacity in several hepatoma cell lines. Low level of forced c-MYC expression also markedly (10–100-fold range) increased the percentage of SP cells and promoted tumorigenicity underscoring the c-MYC ability to significantly modulate the key CSCs properties, such as self-renewal and tumor-initiation potential (35). Consistent with this, we recently demonstrated that c-MYC activation is a key requirement for oncogenic reprogramming of hepatic lineage cells including fully differentiated hepatocytes into CSC (17). However, the results of this study do not allow discriminating whether forced expression of c-MYC activated the preexisting CSCs or caused a de novo generation of CSCs from a more differentiated progeny.

Mechanistically, the CSC-promoting effects of c-MYC were linked to p53 status in the investigated hepatoma cells. p53 is known to enhance differentiation in embryonic stem cells by inhibiting NANOG expression via promotor binding (36). The evidence is growing that besides inhibition of cell cycle and induction of apoptosis, tumor-suppressor p53 may also function as a major gatekeeper of self-renewal by restricting self-renewing divisions, inhibiting symmetric division and blocking reprogramming of somatic/progenitor cells into stem cells (32, 37, 38). Accordingly, the biggest impact on liver CSC
properties induced by forced c-MYC expression was achieved in p53-null Hep3B. Furthermore, reducing p53 signaling by p53 knockdown in HepG2 cells increased the self-renewal potential only in the context of c-MYC activation. In a similar way, shRNA-mediated silencing of endogenous p53 in liver-specific c-MYC transgenic mice accelerated tumorigenility only in the presence of c-MYC activation (39). Also, it was recently reported that knockdown of p53 in ovarian cancer cells by RNAi triggered the expansion of EpCAM-expressing stem-like tumor cells, promoting their aggressiveness (40). Hence, coordinated downregulation of p53 function and activation of c-MYC signaling could contribute to expansion of hepatic CSC pool and tumor growth. These findings are consistent with a proposed role for p53 in regulation of stem cell homeostasis (41) and underscore a broad regulatory role of the c-MYC gene and its ability to cooperatively drive hepatocarcinogenesis via a variety of oncogenic partners.

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

References
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