

Glypican-3-Mediates Autophagy and Promotes Self-Renewal and Tumor Initiation of Hepatocellular Carcinoma Cells

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Abstract

Objective: Understanding the biological functions of cancer stem cells (CSCs) provide new avenues for therapeutic interventions, which is especially important in hepatocellular carcinoma (HCC), an often fatal malignancy. We aim to determine whether glypican-3 (GPC3), an over-expressed membrane protein in HCC, mediates CSC properties in HCC cells.

Design: We determined the cell surface expression of GPC3 in HCC patients and HCC cell lines, and isolated GPC3-high/low sub-populations to study their abilities to self-renew. Additionally, we used HCC cell-based systems where GPC3 expression was either suppressed or induced, to validate the stem-like properties (spheroid formation, cell cycle progression, tumor initiation) that may be mediated by GPC3.

Results: We observed highly specific cell surface expression of GPC3 in HCC cells only (and not in normal hepatocytes or tumor-associated fibroblasts). The GPC3-high sub-populations isolated from HCC cells possess higher levels of self-renewing ability, have lower percentages of cells in the G₀/G₁ phase, and promoted tumor formation *in vivo*. These observations were confirmed in HCC cell-based systems where GPC3 expression was either suppressed or induced. The effects of GPC3 (and EpCAM and CD133) on spheroid formation and cell cycle were nullified by the starvation-induced autophagy inhibitor, 3-methyladenine (3-MA), indicating that these processes are partially regulated by autophagy.

Conclusion: We provide first evidence that GPC3 is a novel CSC marker in HCC, and that it mediates self-renewal, cell cycle progression, and tumor formation partly *via* autophagy induction. We also suggest that autophagy inhibition may be a general approach for intervening with liver CSC functions, regardless of cell surface marker expression.

Keywords: Glypican-3; Hepatocellular carcinoma; Autophagy; Self-renewal; Tumor initiation

Abbreviations: HCC: Hepatocellular Carcinoma; GPC3: Glypican-3; PI: Propidium Iodide; NH: Normal Hepatocytes; TAFs: Tumor-Associated Fibroblasts

Introduction

Hepatocellular carcinoma (HCC) is seventh leading cause of cancer worldwide and second in terms of cancer mortality [1]. Emerging evidence indicate that HCC might originate from both adult hepatocytes and hepatic progenitor cells, providing the crosstalk between normal stem cells and cancer stem cells (CSCs) [2,3]. Self-renewing HCC cells may arise from either normal stem cells or more differentiated cells by infection of hepatitis virus (HBV or HCV) or oncogenic mutations that disrupt the normal developmental pathway [4,5]. CSCs are considered the root of cancers, as they self-renew, undergo aberrant differentiation, and are therefore tumorigenic [6]. They are also proposed to be resistant to conventional chemo- and radiotherapies, allowing them to persist and cause relapse and metastasis. CSC phenotypes can vary among different patients, and multiple distinct CSC populations can exist in a single tumor [7]. The ability to specifically target multiple CSCs for the treatment of cancer can potentially improve the survival of cancer patients [8]. HCC would particularly benefit from CSC-targeted therapies due to its aggressiveness and inherent resistance to currently available therapies [9,10]. Therefore, an understanding of the liver CSC biology, and identification of liver CSC markers for delineating their signaling pathways would potentially impact on the clinical management of HCC patients.

To date, several liver CSC markers have been identified, including

CD90, CD133, EpCAM, CD13, and CD24 [11-15]. We propose that GPC3 is a novel and biologically relevant liver CSC marker. Glypican-3 (GPC3) is a cell surface-linked heparan sulfate proteoglycan that is highly expressed during embryogenesis. In the fetal liver, GPC3 expression gradually decreases towards birth, and increases again in activated adult liver progenitor cells and HCC [16,17], implicating it as a marker of hepatic progenitor cells and of early liver lesions. Indeed, GPC3 is now established as a serum and histological marker of HCC [18], and is reported to modulate important biological functions in HCC cells via the Wnt signaling pathway, which is involved in the formation and maintenance of CSCs [19]. As yet, there is no direct evidence of GPC3 as a liver CSC marker or that it supports liver CSC functions. Here, we provide the first documentation that GPC3 promotes self-renewal and subsequent tumor initiation of HCC cells, which may be mediated by GPC3-induced autophagy. Additionally, we show that targeting multiple CSC populations by inhibiting autophagy is a feasible strategy to inhibit the self-renewal of HCC cells.

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Materials and Methods

Isolation of single cell suspensions from HCC tumor samples

This study was approved by the Stanford Institutional Review Board. From May 2007 to September 2012, 10 patients diagnosed with HCC that were resectable without preoperative adjuvant therapy were included in this study. Informed consent was obtained prior to surgery. Patient tumors and their corresponding non-tumorous liver were collected by Dr. Samuel So and surgeons from Stanford Liver Transplant Clinic.

Tumor tissues were minced and digested in Hank's Balanced Salt Solution (HBSS) supplemented with 5% BSA, 50 mM HEPES, Fungizone (Life Technologies, Grand Island, NY), collagenase type IV (1 mg/ml), DNase I (20 mg/ml), and hyaluronidase (100 µg/ml) (Sigma-Aldrich, Missouri, MO) at room temperature for 2 hours. Afterwards single cell suspensions were obtained by passing the digestion media through a 75 µm nylon mesh (BD Falcon, San Jose, CA), centrifuged at 700 rpm for 45 sec and resuspended in BEBM™ BulletKit™ medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY) for subsequent experiments.

Generation of stable, doxycycline-inducible GPC3-expressing HCC cell lines

GPC3 full length cDNA containing the signal peptide was generated by RT-PCR from HepG2 mRNA, and then cloned into the lentiviral vector pLVX-Tight (Clontech, Mountain View, CA). Recombinant lentivirus was produced using Trans-Lentiviral Packaging Kit (Thermo Scientific, Pittsburgh, PA). HCC cells (SNU-449, SNU-398, PLC/PRF/5) were then co-transduced with the recombinant lentivirus pLVX-Tet-On Advanced (Clontech, Mountain View, CA) and pLVX-Tight encoding the full length GPC3 protein according to the manufacturer's protocol. Transduced cells were selected by G418 (600 µg/ml) and puromycin (3 µg/ml). GPC3 expression was induced with doxycycline (0.5 µg/ml) in culture media for 24 h (minimum time needed for induction) prior to experiments, and GPC3 induction confirmed by Western Blotting and FACS analysis.

Spheroid formation assay

Single cell suspensions from HCC patient tumors or HCC cell lines were maintained in serum free "stem cell" media for enrichment of self-renewing cells [20]. Medium was prepared by using DMEM/F-12, GlutaMAX™ media supplemented with human recombinant insulin (5 µg/ml), hrEGF (20 ng/ml), bFGF (10 ng/ml), 0.4% BSA, 100 U/ml penicillin and 100 µg/ml streptomycin (all media and reagents from Life Technologies, Grand Island, NY) in 6-well ultra-low attachment plates (Corning, Tewsbury, MA). Culture medium was changed every 3 days for 2 weeks. Tumor spheres were counted under a light microscope. Serial passage of tumor spheres were performed by digestion in liver tumor digestion media as described above. The percentage of self-renewing cells was determined by calculating the number of spheroids formed/the number of cells seeded (typically 20,000 cells/well), and is based on the assumption that each spheroid is formed by a single cell. Cell viability was confirmed by counting cells in 0.2% trypan blue (Life Technologies, Grand Island, NY) and cells were seeded in fresh sphere culture media. Three independent experiments were done, each in triplicates.

Experimental induction/inhibition and analysis of autophagy

Cells (2×10^5) were seeded in 6-well plates in 2 ml of cell culture

media supplemented with 10% FBS. Starvation-induced autophagy was inhibited by culturing cells in media supplemented with 5-10 mM of 3-methyladenine [21] (3-MA, Sigma-Aldrich, Missouri, MO) for 24 hours before initiation of experiments. Starvation induced-autophagy was induced by washing the cells in 1X PBS, followed by culturing in Krebs-Ringer buffer (Sigma-Aldrich, Missouri, MO). Total cell lysates were collected at specific time points for immunoblotting. For detection of autophagosomes, immunofluorescence staining of the cultured cells fixed in 4% paraformaldehyde (PFA)/PBS was performed using anti-LC3B antibody (Cell signaling Technology, Danvers, MA).

Inoculation of mice with HCC cells

Animal studies were carried out in compliance with Federal and local institutional rules for the conduct of animal experiments. Single cells labeled with specific antibodies were sorted by using BD FACS ARIA II (BD BioSciences, Franklin Lake, NJ), then counted and resuspended in 100 µl of BEBM™ BulletKit™ medium. Cell suspensions were then mixed with equal volumes of matrigel and injected subcutaneously into 4 week old NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice by using a 22 gauge needle (BD Falcon, San Jose, CA). Subsequently we used 100 cells per injection as the minimum of cells required for tumor initiation, as determined by serial dilution studies. Tumor growth was monitored 3 times per week.

Statistical analysis

Statistical analyses were done using Microsoft Excel (Microsoft, Redmond, Washington). The critical value table was used to calculate the Pearson's *r* linear correlation factor and *P* values comparing the percentage of spheroid forming cells and their corresponding cell surface marker expression (determined by flow cytometry) (Table 1). Statistical significance for other experiments was determined by independent-samples *t*-test. *P* values < 0.05 and < 0.01 were considered statistically significant and highly significant, respectively.

Results

GPC3 is a HCC specific cell surface marker with positive correlation to self-renewal

Using flow cytometry, GPC3 cell surface expression was observed predominantly in HCC cell lines (0.23 to 77.37%; *n*=9) and HCC patient tumors (0.5 to 6.39%; *n*=10) only, and negligible in normal hepatocytes (NHs) (0.1 to 0.76%; *n*=10) and tumor-associated fibroblasts (TAFs) (0.05 to 1.24%; *n*=8) (Figure 1A, Supplemental Table S3 to S6). By using 1% above the baseline as a cut-off to distinguish positive cells from possible background staining, 7 out of 10 (70%) HCC patient

	HCC Cell lines		HCC patient tumor		
	Pearson's <i>r</i>	<i>P</i> value	Pearson's <i>r</i>	<i>P</i> value	
GPC3	0.649	0.058	GPC3	0.709735	0.014
CD24	0.236	0.54	CD44	0.425302	0.192
CD13	0.063368	0.871	CD90	0.373533	0.257
CD133	0.056168	0.885	CD271	0.220595	0.514
EpCAM	-0.03163	0.935	EpCAM	0.111829	0.743
CK19	-0.05059	0.897	CK19	0.011358	0.973
CD47	-0.05086	0.986	CD47	-0.00793	0.981
CD44	-0.30835	0.42	CD24	-0.0378	0.912
CD90	-0.35444	0.349	CD13	-0.04706	0.89
CD271	-0.48172	0.189	CD133	-0.14798	0.664

Table 1: Correlation (Pearson's *r*) of cell surface marker expression with their percentage of spheroid forming cells from HCC cell lines or HCC cells from HCC patient tumors.

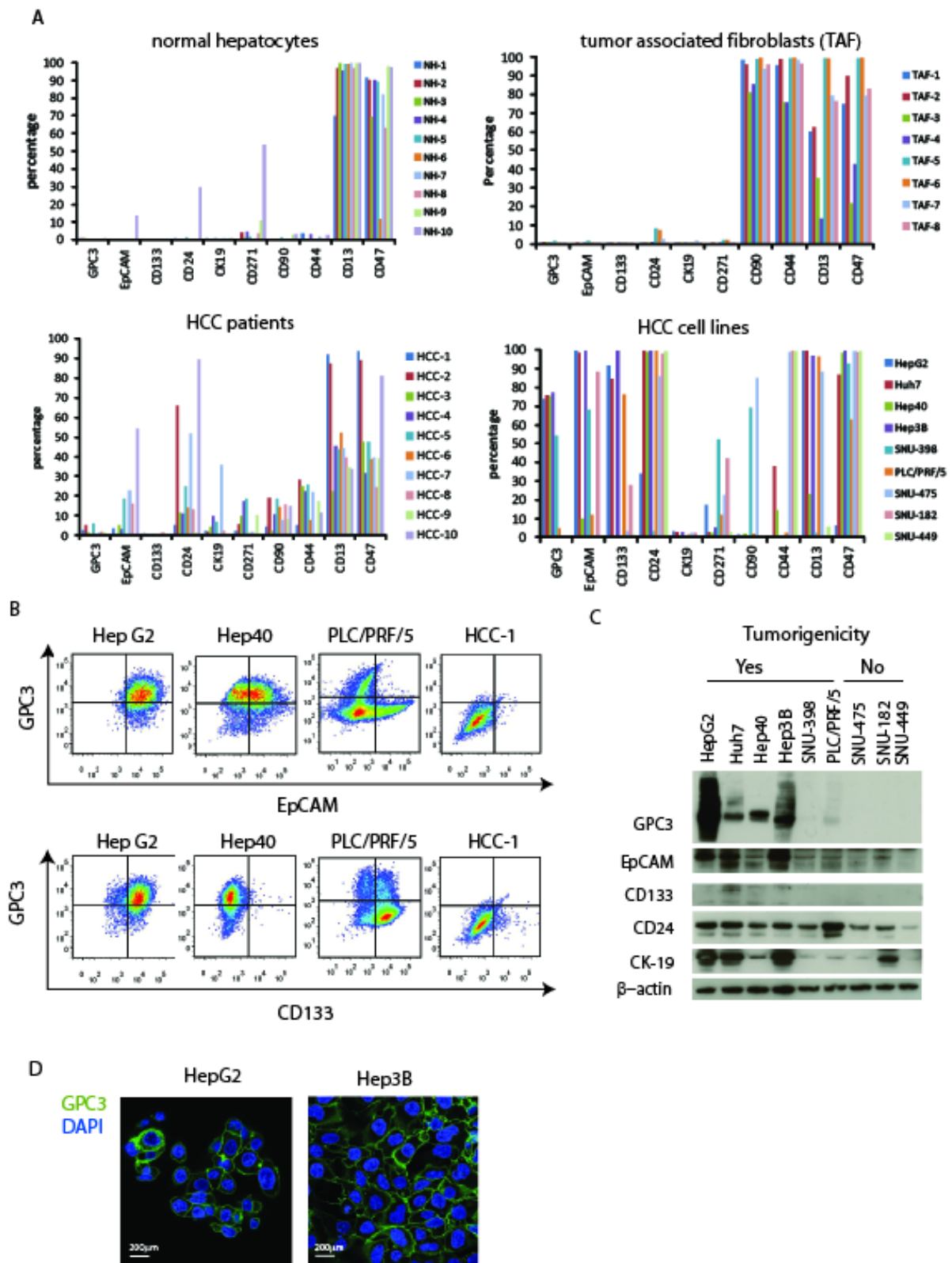


Figure 1: GPC3 expression in HCC cells and its correlation with self-renewal. A. Expression profiling of liver CSC markers by flow cytometry in normal hepatocytes (NH), tumor associated fibroblasts (TAFs), and malignant hepatocytes isolated from HCC patients and HCC cell lines. B. Two-color flow cytometry analysis on the co-localization of cell surface expression of GPC3 with CSC markers EpCAM and CD133. C. Expression of GPC3, EpCAM, CD133, CD24, and CK-19 proteins in HCC cell lines with different tumorigenic potentials. D. Immunofluorescence staining of GPC3 (green) and DAPI (blue) showing cell surface location of GPC3 in HepG2 and Hep3B cells (Magnification=400X).

tumors showed more than 1% GPC3 positive cells, in agreement with an earlier histological study on the over-expression of GPC3 in HCC tumors [22]. Liver CSC markers such as CD271, CD90, CD44, CD13, and CD47 were present in NHs and/or TAFs as well, whereas EpCAM, CD133, CD24, and CK19 showed differential expressions in HCC cell lines and/or HCC patient tumors when compared to NHs and TAFs. Two color flow cytometry demonstrate that GPC3 does not necessarily co-localize with other markers, since multiple GPC3, EpCAM, or CD133 single positive sub-populations exist among HCC cell lines and one HCC patient sample (HCC-1, Figure 1B).

Among GPC3, EpCAM, CD133, CD24, and CK-19, only the expression pattern of GPC3 in HCC cell lines correlated with *in vivo* tumorigenicity (four out of six tumorigenic cell lines express high levels of GPC3, Figure 1C). All three non-tumorigenic cell lines express no detectable levels of GPC3, although they have detectable expression of EpCAM, CD24 and CK-19. Cell surface expression of GPC3 was confirmed by immunofluorescence in GPC3-positive cell lines (Representative images shown in Figure 1D). The dye retaining population within xenografts established from Huh7, Hep3B, and PLC/PRF/5 cells contained a higher percentage of GPC3+ cells, confirming their stem-like property (Supplementary Figure S1A).

Spheroid formation assay demonstrated the presence of self-renewing sub-populations of cells in HCC cell lines and HCC patient tumors (Supplementary Figure S1B). Additionally, the number of spheroids formed correlated significantly with the percentage of cell surface expression of GPC3 (detected by flow cytometry) in patient tumors (Pearson's $r=0.709$, $P=0.014$) and almost statistically significant in HCC cell lines (Pearson's $r=0.649$, $P=0.058$) (Table 1). These results have shown the specificity of GPC3 on the cell surface expression of HCC tumors/cell lines and its correlation with self-renewal and tumorigenicity in mice.

GPC3 is a critical regulator of self-renewal in HCC cells

GPC3-high and GPC3-low sub-populations were isolated from Huh7 and Hep40 cells by flow cytometry, and GPC3 expression in these sub-populations confirmed by Western blotting and flow cytometry (Figure 2A). Spheroid formation assay demonstrated that self-renewing cells can be enriched in the GPC3-high sub-population compared to the GPC3-low cells (Figure 2A). When GPC3 expression was suppressed in Huh7 and Hep40 cells using siRNA against full-length GPC3 (Supplementary Figure S2A), the percentage of spheroids formed were significantly reduced, compared to control siRNA-transfected cells for both cell lines (Figure 2B; left panel $P<0.005$). Additionally, spheroid forming ability of Huh7 and Hep40 cells were decreased when co-treated with 3-MA (a starvation-induced autophagy inhibitor) (Figure 2B right panel, $P<0.005$). Our observations indicate possible roles of GPC3 and starvation-induced autophagy on the regulation of self-renewal. When GPC3 expression was induced in the GPC3-null HCC cell line (SNU-449), and two GPC3-low HCC cell lines (SNU-398 and PLC/PRF/5) (Figure 2C), we observed significantly increased numbers of spheroids compared to no-doxycycline (GPC3-) controls in all three cell lines (Figure 2D, $P<0.005$). GPC3-transduced cells proliferated at similar rates as their no-doxycycline controls; excluding the possibility that enhancement of spheroid formation is caused by increased cell proliferation (Supplementary Figure S2B).

GPC3 mediates self-renewal through inducing autophagy in HCC cells

Based on our above observations (Figure 2B) and a previous

report that autophagy is necessary for stem cell maintenance [23], we hypothesized that autophagy might be involved in GPC3-mediated self-renewal. We identified the presence of more autophagic vesicles (with positive staining of LC3B) in SNU-449, SNU-398, and PLC/PRF/5 cells transduced with full length GPC3, compared to un-induced cells (Figure 3A), suggesting a correlation between GPC3 expression and the number of autophagosomes. Altered expressions of autophagy-related genes in SNU-449 cells and its GPC3-transduced counterpart were observed, with up-regulation of ATG5, and down-regulation of P62/HMGA2 in the latter, suggesting the role of GPC3 in the regulation of autophagy (Figure 3B, Supplementary Figure S3). When autophagy was induced in SNU-449 cells transduced with GPC3, we observed enhanced accumulation and clearance of LC3B in a time dependent manner, indicating a complete autophagic process. Inhibition of autophagy by 3-MA prevented the clearance of LC3B protein and resulted in its accumulation, confirming the role of GPC3 in the regulation of autophagy (Figure 3C).

Additionally, co-treatment with 3-MA significantly reduced the number of spheroids induced by GPC3 over-expression in SNU-449, SNU-398, and PLC/PRF/5 cells ($P<0.01$ for all cell lines, Figure 3D), demonstrating the involvement of starvation-induced autophagy in GPC3-mediated spheroid formation.

GPC3 regulates cell cycle progression via autophagy in HCC cells

We observed that GPC3-high sub-populations isolated from Hep40 and Huh7 cells have lower percentages of cells in the G_0/G_1 phase, compared to that in the GPC3-low sub-populations (Figure 4A; $P<0.005$), indicating a possible role of GPC3 in modulating G_1 to S phase progression. Additionally, GPC3-high sub-populations from Hep40 and Huh7 cells have higher levels of cell cycle regulators, such as p15^{INK4B}, p21, cdk2, phosphorylated-cdk2 (p-cdk2), and phosphorylated Rb (p-Rb) than their GPC3-low counterparts (Figure 4B). We also observed up-regulation of autophagic genes ATG5, beclin-1, and LC3B in the GPC3-high sub-populations isolated from Hep40 and Huh7 cells (Figure 4B).

Additionally, induced expression of GPC3 in SNU-449 cells up-regulated the expression of cell cycle regulators p15^{INK4B}, p21, together with enhanced phosphorylation of Rb and cdk2 (Figure 4C). Cell cycle analysis confirmed that GPC3 over-expression promoted cell cycle progression from G_0/G_1 phase to S phase, with decreased percentage of G_1 phase cells, and increased percentage of S phase cells compared to un-induced cells ($P<0.005$ for G_1 and S phase comparisons, Figure 4C). When autophagy was induced experimentally in SNU-449 cells, p21 was up-regulated in no-doxycycline control cells, which was further enhanced in GPC3-transduced cells. 3-MA abolished the up-regulation of p21 mediated by GPC3, indicating that this process may be mediated by autophagy (Figure 4D, Supplementary Figure S3). Pre-treatment of Huh7 and Hep40 cells with 3-MA (for 24 h) increased the percentages of G_0/G_1 populations in both cell lines, indicating a cell cycle arrest at G_0/G_1 phase ($P=0.006$ and 0.001 for Huh7 and Hep40 cells respectively, Figure 4E).

GPC3 is a critical regulator of tumor initiation *in vivo*

In vivo, PLC/PRF/5 cells with induced GPC3 expression showed formed tumors earlier than parental cells and no-doxycycline cells, with no observable difference in the tumor volumes at the end of the experiment (Figure 5A, left panel). Similarly, SNU-398 cells with induced GPC3 formed tumors earlier than control cells, and produced

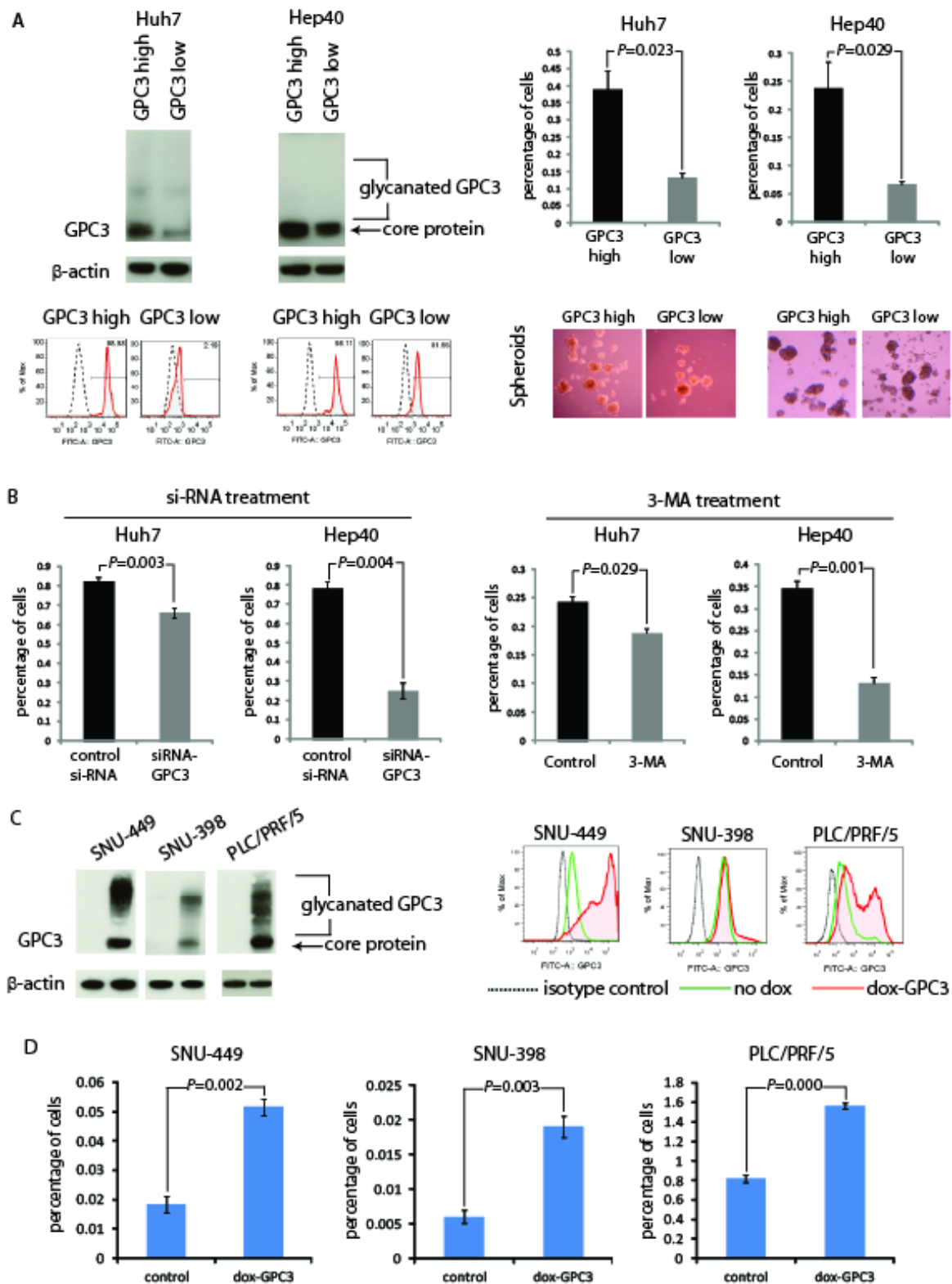


Figure 2: GPC3 is a critical regulator of self-renewal in HCC cells. A. Western blot and flow cytometry were used to confirm GPC3 expression in GPC3-high and GPC3-low sub-populations isolated from Huh7 and Hep40 cells. The percentage of cells that form spheroids are enriched in the GPC3-high sub-populations from both Huh7 and Hep40 cells. B. Left panel-Suppression of GPC3 expression by siRNA down-regulated spheroid formation in both Huh7 and Hep40 cells. Right panel-Effects of 3-MA (10 mM) on spheroid formation in Huh7 and Hep40 cells. C. GPC3 expression was induced in SNU-449, SNU-398, and PLC/PRF/5 cells, and GPC3 levels confirmed by Western blotting. Flow cytometry confirmed cell surface expression of GPC3. D. GPC3 induction in SNU-449, SNU-398, and PLC/PRF/5 cells promoted the percentage of cells that formed spheroids; $P < 0.005$.

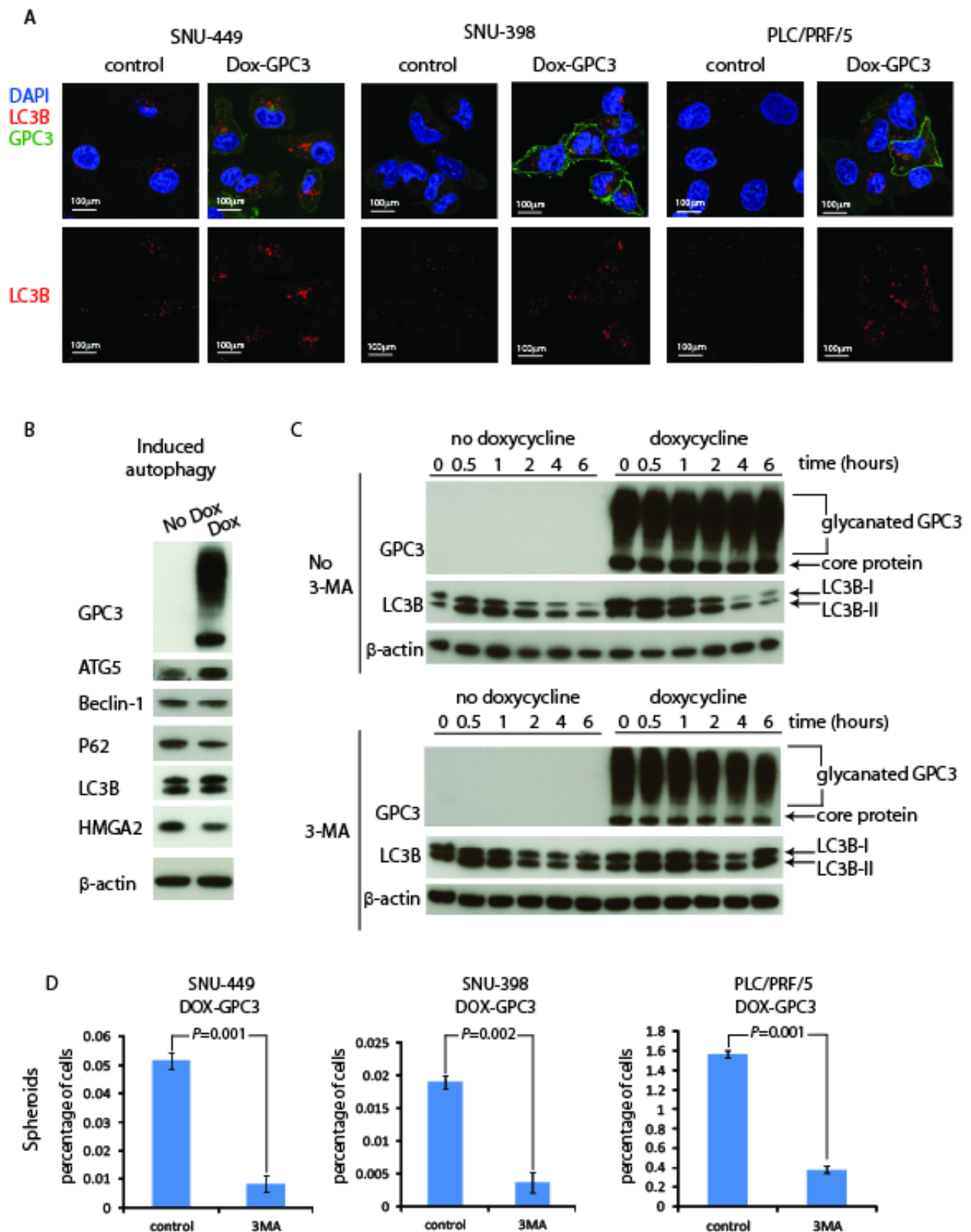


Figure 3: GPC3 mediates self-renewal via induction of autophagy. A. Immunofluorescence of LC3B (red), GPC3 (green) and DAPI (blue) showing the presence of autophagic vesicles in SNU-449, SNU-398, and PLC/PRF/5 cells after GPC3 induction. B. Western blotting for autophagy-related genes upon induction of GPC3 in SNU-449 cells. C. Induction of GPC3 promoted accumulation and clearance of LC3B-II protein in SNU-449 cells (in 'No 3-MA' panel). 3-MA (10 mM) resulted in accumulation (without clearance) of LC3B-II protein in both un-induced SNU-449 cells (GPC3-null) and doxycycline-induced SNU-449 cells (GPC3 positive). D. Treatment of cell lines over-expressing GPC3 with 3-MA down-regulated spheroid formation ($P < 0.005$).

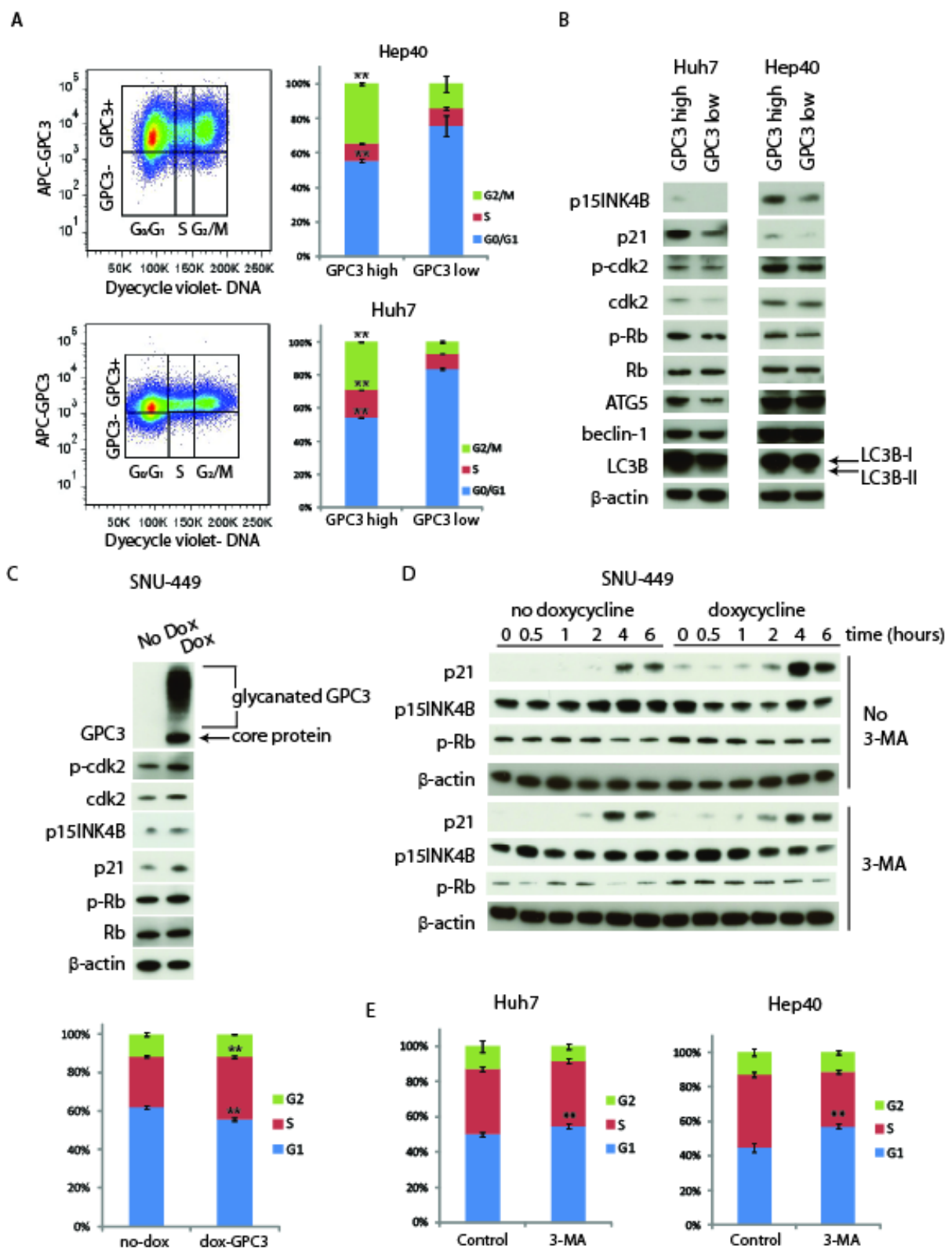


Figure 4: Regulation of cell cycle progression from G₀/G₁ to S phase by GPC3 via activation of autophagy. **A.** Cell cycle analysis of GPC3-high and GPC3-low cells isolated from Huh7 and Hep40 cells. **B.** Western blotting of cell cycle regulators in isolated GPC3-high and GPC3-low sub-populations from Huh7 and Hep40 cells. **C.** Protein expression of cell cycle regulators and cell cycle analysis of SNU-449 cells with un-induced (no-Dox), or induced (Dox) GPC3. **D.** Effects of experimental induction of starvation-induced autophagy and its inhibition by 3-MA (10 mM) on the protein levels of p21, p15^{INK4B} and phosphorylation of Rb in SNU-449 cells with un-induced (no-Dox), or induced (Dox) GPC3. **E.** Effects of 3-MA on cell cycle progression in Huh7 and Hep40 cells as analysed by flow cytometry.

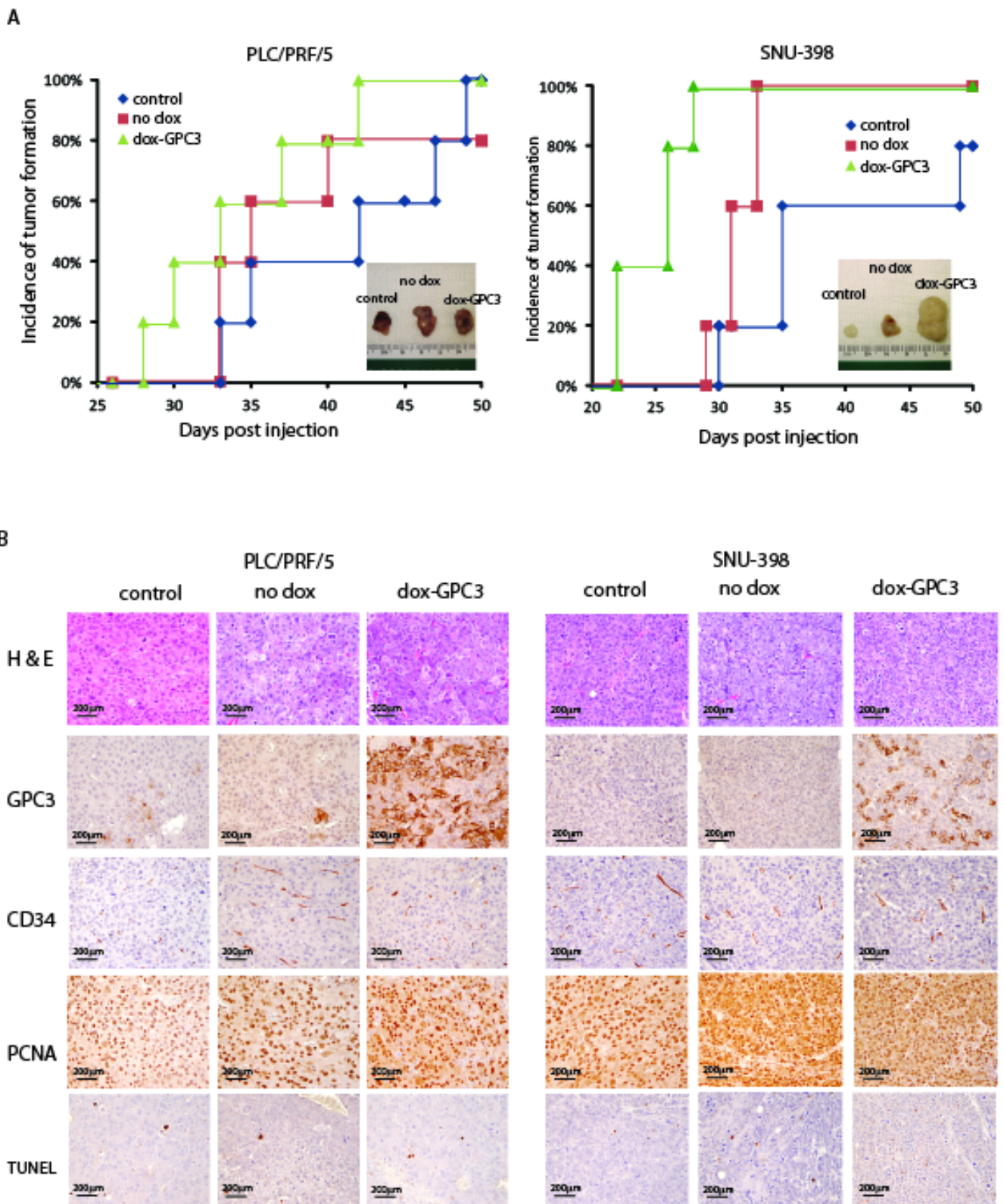


Figure 5: GPC3 promotes tumor initiation *in vivo*. A. Effects of induced GPC3 expression in PLC/PRF/5 and SNU-398 cells on tumor initiation in NSG mice (~100 cells were inoculated subcutaneously; n=5 for each group), and GPC3 expression was induced by adding doxycycline in the drinking water. Tumors were harvested at the end of the experiment (insets). B. Immunohistochemistry showing GPC3 expression (GPC3), cell morphology (H&E), tumor angiogenesis (CD34), cell proliferation (PCNA), and apoptosis (TUNEL) in respective tumors established from PLC/PRF/5 and SNU-398 cells with or without induced GPC3 expressions (Magnification=200X).

larger tumors compared to the controls, probably due to earlier tumor initiation rather than enhanced cell proliferation (Figure 5A, right panel). Immunohistochemistry confirmed the induction of GPC3 by doxycycline in xenografts established from GPC3-transduced cells, and showed no observable differences in cell proliferation, angiogenesis, or apoptosis as shown by PCNA, CD34, or TUNEL staining, respectively (Figure 5B).

GPC3+ cells isolated from PLC/PRF/5 cells were able to form tumors *in vivo*, regardless of growth factor supplementation (whether regular matrigel with high concentration of growth factors, or growth factor reduced matrigel was used) (Supplemental Figure S4A,B). This indicates that the tumor initiating ability of GPC3 is nutrient independent. Onset of tumor initiation was delayed when the GPC3+ sub-populations were pre-incubated with 3-MA and mixed with matrigel with high concentration of growth factors prior to injection into mice (Supplemental Figure S4C), showing that promotion of tumor initiation by GPC3 is autophagy dependent.

3-MA inhibits biological functions of CSCs in HCC cells

We isolated GPC3+, EpCAM+, and CD133+ sub-populations from PLC/PRF/5 cells and compared their stem-like properties. Among the three sub-populations, GPC3+ cells contained the most number of double membrane autophagic vesicles as observed by transmission electron microscopy and confirmed by positive LC3B immunofluorescence staining (Figure 6A). They also have the highest expression of autophagic protein LC3B-II and the lowest expression of senescence associated protein HMGA2 (Figure 6B), indicating a stronger autophagic activity. The GPC3+ sub-population also contained the highest percentage of spheroid-forming cells compared to the EpCAM+ or CD133+ sub-populations ($0.627 \pm 0.003\%$ for GPC3+, $0.1575 \pm 0.002\%$ for EpCAM+, and $0.1775 \pm 0.002\%$ for CD133+; $P < 0.001$ for GPC3+ vs. EpCAM+ and CD133+). All three sub-populations formed secondary and tertiary spheres with enrichment of self-renewing cells upon serial passage (Figure 6C). Despite these variations in self-renewing ability, all three sub-populations showed down-regulation of spheroid formation ($P < 0.01$, Figure 6C) with concomitant accumulation of LC3B-II protein when treated with 3-MA under nutrient deprived media, with greatest effects were seen in the GPC3+ sub-population (Figure 6D).

The GPC3+ sub-population had a higher percentage of S phase cells ($P < 0.001$ vs. EpCAM+, $P = 0.007$ vs. CD133+, Figure 6E), and the highest expression of cell cycle regulators p15, cyclin D1, and phosphorylated cdk2 than the EpCAM+ or CD133+ sub-populations (Figure 6B). Treatment with 3-MA significantly increased the percentage of GPC3+ cells in the G_0/G_1 phase, even at low dose (5 mM); however, significance was achieved in EpCAM+ and CD133+ sub-populations only at a higher dose of 3-MA (10 mM) (Figure 6F). Thus, inhibition of autophagy could induce cell cycle arrest of liver CSCs, regardless of cell surface marker expression.

Additionally, the GPC3+ sub-population showed the strongest ability to initiate tumor formation (compared to the CD133+ and EpCAM+ sub-populations) in NSG mice when growth factor-reduced matrigel was used (Supplementary Figure S4). Tumor initiation by all sub-populations was delayed by pre-incubation with 3-MA, indicating dependence on autophagy (Supplementary Figure S4).

Discussion

We provide new evidence that GPC3 is a marker of liver CSCs, and that GPC3+ HCC cells possess stem-like properties, being able to self-renew and initiate tumor formation. GPC3 appears to modulate these

properties *via* the induction of autophagy, an evolutionarily conserved cellular process [24] that is especially important in stem cells, due to their long life span, limited capacity to dilute cellular waste and spent organelles to due quiescence, and their requirement for remodeling in order to differentiate [25]. Indeed, for development of new tumors, autophagy is critical in providing nutrients from within the cells for a limited period of time until external nutrients are available [26,27]. Using different cell-based models (GPC3 induction or suppression, and isolation of GPC3+/GPC3-high sub-populations), we demonstrated that GPC3+ cells possess enhanced spheroid formation *in vitro* with higher levels of autophagy associated proteins. This enhanced self-renewal ability can be nullified by the starvation-dependent autophagy inhibitor 3-MA. Similarly, GPC3+ sub-populations promoted *in vivo* tumor formation under nutrient-deprived conditions, which may also be associated with their higher levels of autophagy. Recently, the up-regulation of GPC3 and P62 in HCC tumors was associated with a defective autophagic mechanism in HCC cells [28]. This might result from loss of autophagic activity upon differentiation of GPC3+ cells, differences in genetic contexts, and the presence of other sub-populations (EpCAM+ and CD133+). Further studies are needed to delineate the role of GPC3 and P62 in cell differentiation and autophagy regulation.

We also demonstrate that GPC3 promotes cell cycle progression from the G_0/G_1 to S phase, with GPC3+ cells having a lower percentage of G_0/G_1 phase cells and a higher percentage of S phase cells, with concomitant up-regulation of cdk2 phosphorylation, which regulates G_0 to S phase transition. Our observations are consistent with the notion that pluripotent cells spend longer time in the S phase and shorter time in the G_1 phase compared to somatic cells [29], and with our earlier report that GPC3 suppression induced cell cycle arrest at the G_1 phase [30]. Induction of autophagy in GPC3-transduced cells resulted in cell cycle re-entry in the S phase, together with changes in the levels of cell cycle regulators that regulate G_1 to S phase transition and subsequent self-renewal (such as transient inactivation of p15^{INK4b}, up-regulation of p21 expression, and Rb phosphorylation) [31]. Autophagy inhibition by 3-MA prevented cell cycle re-entry at the S phase, resulting in G_1 phase arrest and suppressed self-renewal of HCC cells. Our data indicate that G_1 to S phase transition and the subsequent self-renewing property of GPC3+HCC cells are in part mediated via autophagy.

Cancer heterogeneity caused by genetic mutations, epigenetic mechanisms, or interaction of cancer cells with their surrounding environment [7] may contribute to the differences in phenotypes, functions, and responses to therapy [32]. It therefore appears necessary to target all CSC sub-populations in order to achieve satisfactory clinical outcome [33]. Previous reports have characterized multiple CSCs in HCC by focusing on different properties of the stem cell sub-population, including side population, resistance to chemotherapy, and tumor initiation [11-15,34]. Here, we show that some of the markers might lack specificity in HCC cells owing to their presence in other cell types (NHs, TAFs). Additionally, technical differences in the sampling and processing of HCC tumors may contribute to isolation of different sub-populations. We demonstrated that multiple liver CSC sub-populations (GPC3+, CD133+ and EpCAM+) can be isolated from a single cell line, with each sub-population exhibiting observable differences in self-renewing abilities and cell cycle characteristics. Despite these differences, treatment with 3-MA successfully inhibited self-renewal and spheroid formation of GPC3+, EpCAM+, and CD133+ sub-populations. Indeed, CD133+ liver CSCs were shown to modulate HCC cell growth via regulation of autophagy [35]. Additionally, CD90+ liver CSCs were reported to over-express GPC3 [36], highlighting possible shared biological properties of CD90+ and

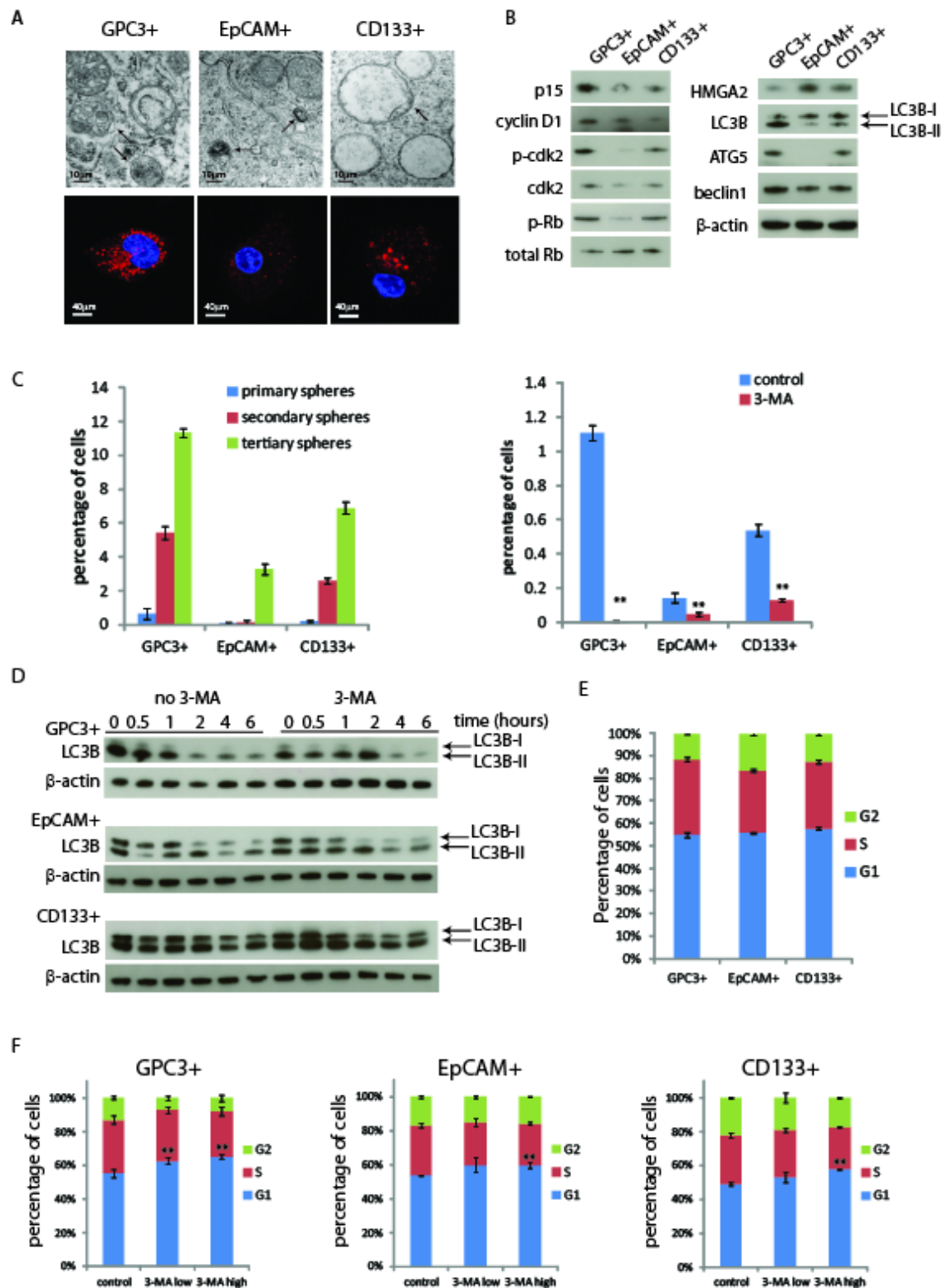


Figure 6: The effects of 3-MA on self-renewal and cell cycle progression of liver CSCs. A. TEM and confocal imaging showing the presence of autophagic vesicles in GPC3+, EpCAM+, or CD133+ sub-populations isolated from PLC/PRF/5 cells. B. Western blotting for cell cycle regulators (left) and autophagic regulators (right) in GPC3+, EpCAM+, or CD133+ sub-populations isolated from PLC/PRF/5 cells. C. Spheroid formation assay showing the enrichment of self-renewing populations by serial passage in GPC3+, EpCAM+, or CD133+ sub-populations, and the significant reductions in spheroid forming cells after treatment with 3-MA in each sub-population ($P < 0.01$ for all sub-populations). D. Western blotting showing the effects of 3-MA on the accumulation and clearance of LC3B protein in these sub-populations. E. Cell cycle analysis and F. The effects of 3-MA (low dose- 5 mM, high dose-10 mM) on cell cycle progression of the sub-populations isolated from PLC/PRF/5 cells.

GPC3+ liver CSCs. These observations together suggest that targeting shared properties (regulation of autophagy) can inhibit the pro-survival functions mediated by these different sub-populations, to achieve more efficacious eradication of liver CSCs. Moreover, inhibition of autophagy may sensitize cancer cells towards conventional chemotherapy, offering additional benefits when these are used in combination [25]. For example, we observed that 3-MA alone did not significantly alter proliferation of Huh7 and Hep40 cells, but a synergistic inhibitory effect was observed when combined with low dose cisplatin or TGF β 2 (Supplementary Figure S5).

Taken together, GPC3+ HCC cells possess stem-like properties and mediate self-renewal and tumor initiation, likely via modulating autophagy. These new findings further enhance our understanding of the functions of GPC3 in HCC, and provide greater insights into possible ways of targeting this protein for therapeutic purposes. Specifically, inhibition of autophagy is a feasible approach to inhibit these novel functions of GPC3 (and of GPC3+ cells) in HCC. More broadly, the targeting of autophagy may be a common strategy to inhibit the functions of therapy-resistant CSCs, which may be particularly beneficial for HCC treatment.

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