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Knockdown of GPC3 Inhibits the Proliferation of Huh7 Hepatocellular Carcinoma Cells Through Down-Regulation of YAP

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ABSTRACT

Glypican-3 (GPC3), a membrane-associated heparan sulfate proteoglycan, is frequently upregulated in hepatocellular carcinoma (HCC). Yes-associated protein (YAP) is also found over-expressed in HCC and has been identified as a key effector molecule in Hippo pathway, which could control the organ size in animals through the regulation of cell proliferation and apoptosis and plays an important role in the development of malignant tumors. Studies have reported that GPC3 and YAP might collaborate to regulate the development of HCC. To elucidate the role of GPC3 in the development of HCC and its relationship with YAP, siRNA technique was employed to knock down GPC3 in Huh7 HCC cells. Moreover, recombinant human YAP-1 was used to examine the effects of GPC3 on Huh7 cells. The results of flow cytometric analysis and Annexin-V-FLUOS apoptosis assay showed that knockdown of GPC3-induced apoptosis in Huh7 cells, resulting in inhibition of cell proliferation as examined by EdU incorporation assay, migration, and invasion. GPC3 knockdown also suppressed the expression of YAP in mRNA and protein levels, as examined by fluorescence quantitative PCR and Western blot analysis. Moreover, addition of recombinant human YAP-1 effectively rescued the cells from apoptosis triggered by GPC3 knockdown. Taken together, our findings suggest that GPC3 regulates HCC cell proliferation with the involvement of Hippo pathway. J. Cell. Biochem. 114: 625–631, 2013.

KEY WORDS: GPC3; HEPATOCELLULAR CARCINOMA; YAP; HIPPO PATHWAY

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with morbidity and mortality of HCC rank sixth and third, respectively, among all cancers [Parkin et al., 2005]. HCC is characterized by a high degree of malignancy, rapid development, poor prognosis, and high mortality. Surgery is currently the most effective treatment for HCC. However, metastasis and recurrence are usually observed in 60–70% of patients after radical surgical resection. Higher percentage of metastasis and recurrence and poor prognosis have also been recorded in patients treated with local therapy [Parkin et al., 2005]. Therefore, the search for highly sensitive and specific markers for predicting HCC metastasis, recurrence, and prognosis, has kindled great interest of scientist from the field of biology, pharmacy, and medicine.

Glypican-3 (GPC3) is a protein that plays an important role in the occurrence and development of HCC. It has been found highly expressed even in early-stage HCC tissues [Akutsu et al., 2010; Liu et al., 2010c; Yan et al., 2011]. GPC3 is a member of heparan sulfate proteoglycan family proteins and has a basic structure consisting of a core protein, a heparan sulfate chain and glycosylphosphatidylinositol (GPI) [De Cat and David, 2001]. GPC3 could bind to the exocytoplasmic surface of the cell membrane via the GPI anchor and regulate the cell morphology, adhesion, proliferation, migration, survival, and differentiation by receiving signals from receptors on the cell surface [Baumhoer et al., 2008]. Moreover, expression of GPC3 was also found involved in the development of most tissues and organs originating from the mesoderm [Neri et al., 1998]. Many studies have showed that GPC3 demonstrated crosstalk with several

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tumor-related signal pathways, such as Wnt [Capurro et al., 2005], Hedgehog [Capurro et al., 2008], fibroblast growth factor (FGF) [Midorikawa et al., 2003], insulin-like growth factor (IGF) [Cheng et al., 2008; Sakurai et al., 2010], SMAD [Midorikawa et al., 2003], and transforming growth factor-β [Sun et al., 2011]. However, the role of GPC3 in the development of HCC and the underlying molecular mechanisms remain elusive.

Hippo is a signaling pathway discovered in *Drosophila* a few years ago [Huang et al., 2005]. Recent studies have supported the roles of Hippo pathway in regulating the cell size, organ volume, tissue regeneration, and cancer development [Zhao et al., 2010, 2011]. Yes-associated protein (YAP), a key effector molecule that acts in the downstream of the classical Hippo pathway, plays an important role in promoting cell proliferation, inhibiting apoptosis, causing cell loss of contact inhibition, and promoting cell malignant transformation [Overholtzer et al., 2006; Zender et al., 2006; Zhao et al., 2007; Lee et al., 2010; Liu et al., 2010a; Kowalik et al., 2011]. YAP mRNA and protein expression are upregulated in various kinds of human tumors, including human HCC. Changes in YAP expression may initiate and/or promote liver tumor development [Lee et al., 2010; Kowalik et al., 2011]. Thus, YAP serves as an independent prognostic marker of HCC, and GPC3 and YAP may collaborate to play roles in the development of HCC [Xu et al., 2009].

In the present study, to investigate whether the roles of GPC3 in development of HCC is related with Hippo pathway, we knocked down the expression of GPC3 in Huh7 cells and added recombinant human YAP-1, and assayed the apoptosis, proliferation, migration, and invasion levels of the cells.

MATERIALS AND METHODS

CELL CULTURE

The HCC cell line Huh7 was obtained from the China Center for Type Culture Collection and cultured with 10% fetal calf serum containing Dulbecco's modified Eagle's medium (Gibco-BRL). The cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2 in a cell culture incubator.

DESIGN AND SYNTHESIS OF siRNA

siRNA was designed and synthesized by the Shanghai GenePharma Co., Ltd. according to the *GPC3* gene sequence (GenBank Accession No. NM_001164617) as listed in Table I.

siRNA TRANSFECTION

The cells were seed into six-well plate at 1×10^5 cells and incubated for 24 h. SiRNA transfection was carried out following the instruction of Lipofectamine2000 (Invitrogen) with a siRNA concentration of 50 nM/well. After 72 h, the siRNA-transfected cells were analyzed by real-time PCR and Western blotting.

TOTAL RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from the cells using Trizol. The concentration of extracted total RNA was determined by measuring the absorbance at 260 nm. One microgram of total RNA was used for the first-strand cDNA synthesis with RevertAidTM first Strand cDNA Synthesis Kit (Fermentas TM, Germany). First-strand complementary DNA was synthesized using 1 μl of random hexamere and adjusted to a volume of 12 μl using DEPC-treated water. The mix was incubated at 65 °C for 5 min. The following components were added in the incubate order: 4 μl 5× reaction buffer, 1 μl of Ribolock RNaseTM inhibitor (20 U/μl), 2 μl 10 mM dNTP, and 1 μl RevertAid M-MuLV reverse transcriptase, 200 U/μl. The mixes were incubated at 25, 42, and 70°C for 5, 60, and 5 min, respectively. Then, the cDNAs were used for measurement of RNA abundance by quantitative real-time PCR.

TABLE I. siRNA Sequences

Name	Sense/antisense siRNA (5′–3′)	Target
GPC3-homo-714	CCU GUU UCC AGU CAU CUA UTT AUA GAU GAC UGG AAA CAG GTT	716–734
GPC3-homo-647	CCU GAA AGU AUU UGG GAA UUU TT AAA UUC CCA AAU ACU UUC AGG TT	809–829
GPC3-homo-1633	CCA GUG GUC AGU CAA AUU ATT UAA UUU GAC UGA CCA CUG GTT	1,635–1,653
GPC3-homo-1718	GCU CUG GUG AUG GAA UGA UTT AUC AUU CCA UCA CCA GAG CTT	1,789–1,807
GPC3-homo-2134	GUG GUA GCA UAU AGA UUG UTT ACA AUC UAU AUG CUA CCA CTT	2,205–2,223
Negative control	UUC UCC GAA CGU GUC ACG UTT ACG UGA CAC GUU CGG AGA ATT	

FLUORESCENCE QUANTITATIVE PCR

Quantitative PCR was performed using SYBR-Green PCR Master Mix (TOYOBO, Japan), with 100 ng of cDNA contained in 20 μl of reaction mixture. The primer sequences are shown in Table II. The reaction was performed at one cycle of 95°C for 5 min and 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Three independent experiments were conducted for each sample. Data were analyzed by comparing the $2^{-\Delta\Delta C_t}$ value.

WESTERN BLOT ANALYSIS

Total cellular proteins were extracted by incubating cells in lysis buffer. The protein concentrations in the cell lysates were determined by bicinchoninic acid assay (Pierce). SDS–PAGE was done in 8% glycine gels (Bio-Rad) loading equal amount of proteins per lane. After electrophoresis, separated proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat milk in TBST buffer for 1 h. After that, the membranes were incubated with GPC3, YAP, and GAPDH antibodies (Novus Biologicals) at 1:1,000 dilutions in 5% non-fat milk overnight at 4°C, and then anti-rabbit IgG monoclonal antibody conjugated with horseradish peroxidase (Cell Signaling Technology) at 1:2,000 dilution for 1 h at room temperature. Protein bands were detected using the West Femto system (PIERCE).

TABLE II. Primers for Quantitative PCR

Primers	Sequences (5′–3′)
qr-GPC3-F	AGAAACCTTATCCAGCCGAAGAA
qr-GPC3-R	TGGGTCCAACTACTAAGCT
Yap-F	GAACAGTGTGGATGAGATGGATAC
Yap-R	CAGGAATGGCTTCAAGGTAGTC
GAPDH-F	GGTATCGTGGAAGGACTC
GAPDH-R	GTAGAGGCAGGGATGATG

FLOW CYTOMETRIC ANALYSIS

The cell cycle distribution was analyzed by flow cytometry using PI as a fluorescence probe. After siRNA transfection, the cells were trypsinized, washed with PBS and fixed with 70% ethanol overnight at −20 °C. The fixed cells were washed with PBS and stained with PI working solution (1.21 mg/ml Tris, 700 U/ml RNase, 50.1 μg/ml PI, pH 8.0) for 4 h in darkness. The stained cells were analyzed with Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). The proportion of cells in G0/G1, S and G2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were recorded.

ANNEXIN-V-FLUOS APOPTOSIS ASSAY

Cells were collected after transfection for 72 h, and the translocation of phosphatidylserine in treated cells was detected using the Annexin-V-FLUOS staining kit (Roche Applied Science). Briefly, cells were suspended in 500 μl of binding buffer and incubated at room temperature in the dark for 15 min after labeled with 5 μl of Annexin V-fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide. The stained cells were then analyzed by flow cytometry.

5-ETHYNYL-2′-DEOXYURIDINE (EDU) INCORPORATION ASSAY

EdU incorporation assay was performed using an EdU Apollo DNA in vitro kit (RIBOBIO) following the manufacturer's instructions. Briefly, cells were incubated with 100 μl of 50 μM EdU per well for 2 h. After removing the supernatant, the cells were fixed for 15–30 min at room temperature using 100 μl of fixing buffer (4% polyformaldehyde containing PBS). After fixation, the cells were incubated with 2 mg/ml glycine for 10 min followed by washing with PBS. The cells were then permeated with 100 μl/well permeabilization buffer (0.5% Triton X-100 containing PBS) and incubated with 100 μl of 1X Apollo® solution for 30 min at room temperature in the dark. Subsequently, incubation with 100 μl of 1X Hoechst 33342 solution was conducted for 30 min at room temperature in the dark. The samples were washed before being observed under fluorescence microscopy.

TRANSWELL MATRIGEL INVASION ASSAY

Invasion of cells was evaluated by Transwell matrigel invation assay. Briefly, 200 μl of cells after transfection (1×10^6 cells/ml) and 600 μl of the complete medium were added to the upper and lower compartments of the chamber, respectively. After incubation of 48 h, cells migrating to the lower side of the filter were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, stained with crystal violet, and then observed under a confocal microscope.

WOUND HEALING ASSAY

Cells (1×10^6 per well) were seeded in six-well plates and allowed to grow until 70% confluence. The cells were pretreated with Mitomycin C, which inhibits cell division, so that the difference in motility was not affected by differences in cell proliferation rates. The cells were treated as above, and monolayers were then wounded with a pipette tip. After being washed with PBS for three times, images were taken immediately after scratching for various periods of time in the same marked location of the dish. Data were obtained from three independent experiments.

STATISTICAL ANALYSES

Experiments were carried out at least in triplicate and results were expressed as mean ± SD. Statistical analysis was performed using SPSS statistical program version 13 (SPSS, Inc., Chicago, IL). Difference with $^{*}P < 0.05$ or $^{**}P < 0.01$ was considered statistically significant.

RESULTS

SUPPRESSIVE EFFECT OF GPC3 GENE KNOCKDOWN ON THE YAP EXPRESSION

The expression of GPC3 in various HCC cell lines, including Bel7402, SMMC7721, MHCC97L, MHCC97H, HepG2, Huh7, and LM3 cells, as well as HL7702 normal liver cell line, were evaluated by fluorescence quantitative PCR. The results indicate Huh7 exhibited the highest GPC3 expression level (Fig. 1A). Therefore, the Huh7 cells were chosen for further study. Fluorescence quantitative PCR was used to evaluate the ability of different GPC3 siRNAs to silence GPC3 expression in vitro. The results showed that 50 nM GPC3-homo-1633 was the most effective silencer with suppression rate of 74.84% (data not shown). Thus, GPC3-homo-1633 was used in the subsequent experiments for gene knockdown. Our results showed that the expression levels of GPC3 mRNA and proteins in Huh7 cells as measured by fluorescence quantitative PCR and Western blot analysis were significantly suppressed after transfection with GPC3-homo-1633 (Fig. 1B,C). Furthermore, the effects of GPC3 gene knockdown on the expression of YAP were also examined by fluorescence quantitative PCR and Western blot analysis. The results showed that expression of YAP on mRNA and protein levels were effectively inhibited by GPC3 knockdown (Fig. 2). These results suggest that GPC3 may regulate the development of HCC through modulation of YAP expression.

INHIBITION OF CELL PROLIFERATION BY GPC3 KNOCKDOWN THROUGH INDUCTION OF APOPTOSIS WITH THE INVOLVEMENT OF YAP

Resistance to apoptosis is a common characteristic of tumor cells. Therefore, the roles of GPC3 and YAP in HCC development and malignance were further examined by evaluating the effects GPC3 siRNA and recombinant human YAP-1 (rhYAP-1) on cell apoptosis. The results in Figure 3A revealed that knockdown of GPC3 expression significantly induced early phase apoptosis in Huh7 cells. However, addition of rhYAP-1 effectively inhibited the total cell apoptosis induced by GPC3 gene knockdown. Therefore, knockdown of GPC3 expression may trigger Huh7 cell apoptosis through downregulation of YAP. GPC3 and YAP may collaborate to regulate HCC development.

EdU incorporation assay, a sensitive and specific method, was employed to determine the effects of GPC3 and YAP on HCC cell proliferation. The results in Figure 4 shows that the number of EdU-positive cells in GPC3 siRNA-transfected group decreased by 43%

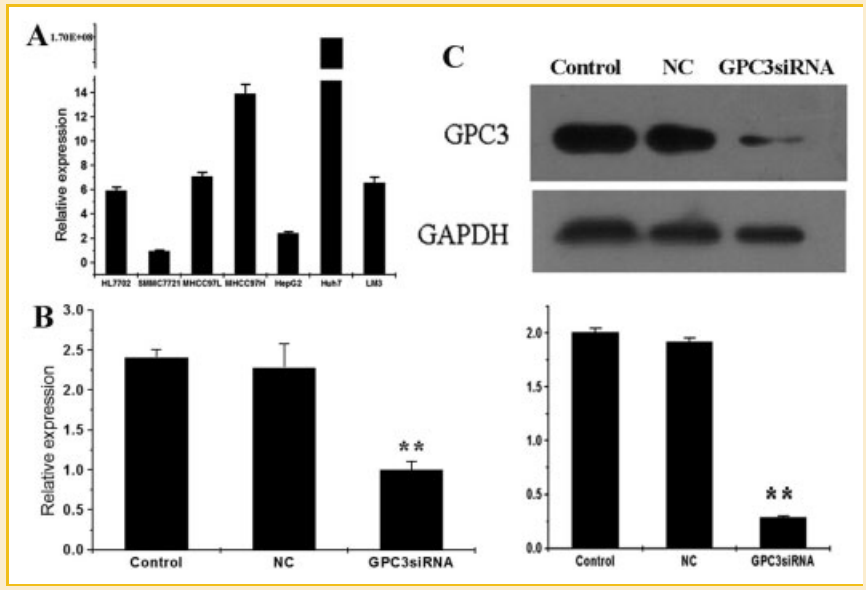

Fig. 1. Expression of GPC3 in various cells and inhibition of GPC3 siRNA. A: Expression of GPC3 in various HCC cells and HL7702 normal liver cells as determined by fluorescence quantitative PCR. B: GPC3 expression in Huh7 cells after transfection with GPC3-homo-1633 as measured by fluorescence quantitative PCR. C: GPC3 expression in Huh7 cells as measured by Western blot analysis. Experiments were carried out at least in triplicate and the results were expressed as mean values, $^{**}P < 0.01$ versus the control. GAPDH serves as a loading control.

compared with the cells transfected with the non-silencer siRNA. However, addition of rhYAP-1 significantly increased the number of EdU positive cells, although still lower than that of non-silencer siRNA group. These results indicate that knockdown of GPC3 could inhibit the proliferation of Huh7 cells, while rhYAP could partly block the effects induced by GPC3 gene knockdown. Taken together, GPC3 knockdown inhibits HCC cell proliferation through induction of apoptosis with the involvement of YAP.

EFFECT OF GPC3 siRNA AND rhYAP-1 ON CELL INVASION AND MIGRATION

Malignant cancer cells can acquire the ability of invasion and metastasis. The transwell assay was used to determine the effect of GPC3 siRNA and rhYAP-1 on HCC cell invasion. The results in Figure 3B showed that Huh7 cell invasion was completely inhibited by GPC3 gene knockdown. In contrast, addition of rhYAP-1 effectively recovered the invasion ability of HCC cells. These results indicated that the expression of GPC3 and YAP play important roles in the invasion of HCC cells.

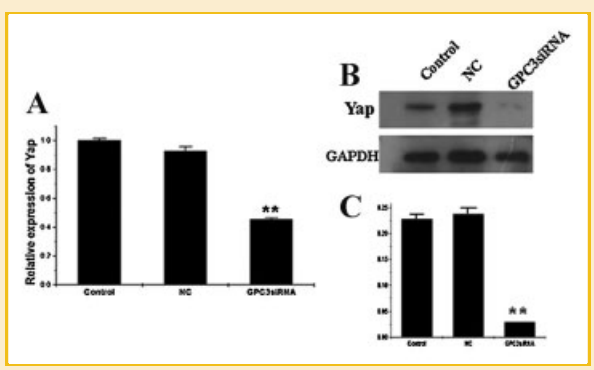

Fig. 2. GPC3 siRNA inhibits the YAP expression. A: YAP expression in Huh7 cells after transfection with GPC3-homo-1633 as measured by fluorescence quantitative PCR. B: YAP expression in Huh7 cells as measured by Western blot analysis. C: Statistical analysis. Each bar represents the mean ± SD from three samples ($^{**}P < 0.01$ vs. the control). GAPDH serves as a loading control.

The scratch wound-healing assay was used to determine the effect of *GPC3* gene silencing on Huh7 cell migration in vitro. The cells were subjected to scratch tests after transfection, and the scratch wound was monitored after different periods of time. The results shown in Figure 5 indicated that the migration of GPC3 siRNA-transfected cells was much slower than that of the NC-siRNA-transfected cells at the indicated time points. Furthermore, addition of rhYAP-1 effectively promoted the cell migration. These results support the important roles of GPC3 and YAP in HCC cell migration.

DISCUSSION

The incidence of primary HCC is about 6% in the world, with over half of which occurred in China [Marrero, 2006]. HCC mortality ranks third among all human cancers, therefore, early diagnosis of HCC was particularly important to extend patient lives [Parkin et al., 2005]. A number of studies have showed that GPC3 was a highly sensitive and specific marker for HCC [Akutsu et al., 2010; Liu et al., 2010a; Yan et al., 2011]. GPC3 expression during human development is highly tissue and stage-specific. GPC3 expression is positive in the liver during the embryonic and fetal periods, but cannot be detected after birth [Iglesias et al., 2008]. Our previous studies (unpublished data) have showed that GPC3 mRNA expression in HCC tissues, paracancerous tissues, and normal liver tissues were 77.1% (37/48), 2.6% (1/39), and 0% (0/31), respectively.

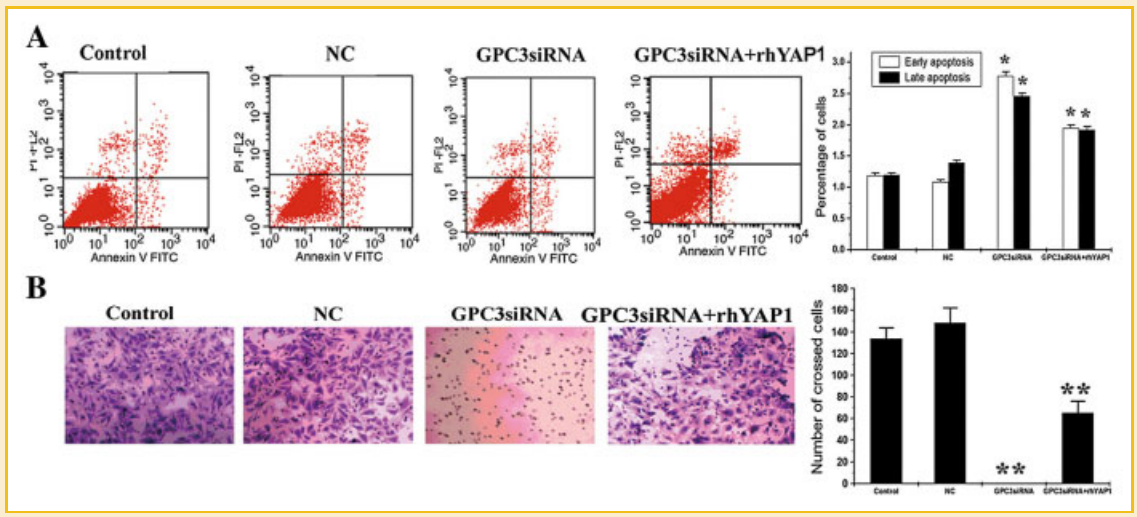

Fig. 3. Effect of GPC3 siRNA and rhYAP on Huh7 cell apoptosis and invasion. A: Huh7 cell apoptosis was measured by Annexin V-FITC-propidium iodide flow cytometric analysis. B: Cell invasion was significantly inhibited after the knockdown of GPC3 expression. NC: NC siRNA was transfected into Huh7 cells; GPC3 siRNA: GPC3 siRNA was transfected into Huh7 cells; GPC3 siRNA + rhYAP: GPC3 siRNA was transfected into Huh7 cells together with the addition of rhYAP-1. The results are presented as mean ($n = 3$) ± SD, $^{*}P < 0.01$ versus the control.

Significant difference between HCC tissues and the other two groups was observed. The expression levels of GPC3 protein were 52.6% (10/19) in small HCC (diameter ≤5 cm) and 86.2% (25/29) in large HCC (diameter >5 cm). Significant difference was also observed between these two groups. The GPC3 expression as measured by Western blot analysis in sera from HCC patients, chronic hepatitis patients, and normal individuals were 51.6% (14/27), 0% (0/20), and 0% (0/8), respectively. Therefore, it could be concluded that GPC3 is of great importance in the development of HCC.

Studies have showed that GPC3 could negatively regulate cell growth under normal circumstances, whereas the mutation and functional loss of the *GPC3* gene resulted in Simpson–Golabi–Behmel syndrome [Pilia et al., 1996], which was characterized by the overgrowth of liver and other organs, increase in the risk of the development of cancers, such as HCC and Wilms tumors [Lapunzina, 2005]. However, the roles and mechanisms of GPC3 in HCC development remain elusive. In the present study, we showed that GPC3 gene knockdown effectively inhibited the HCC cell

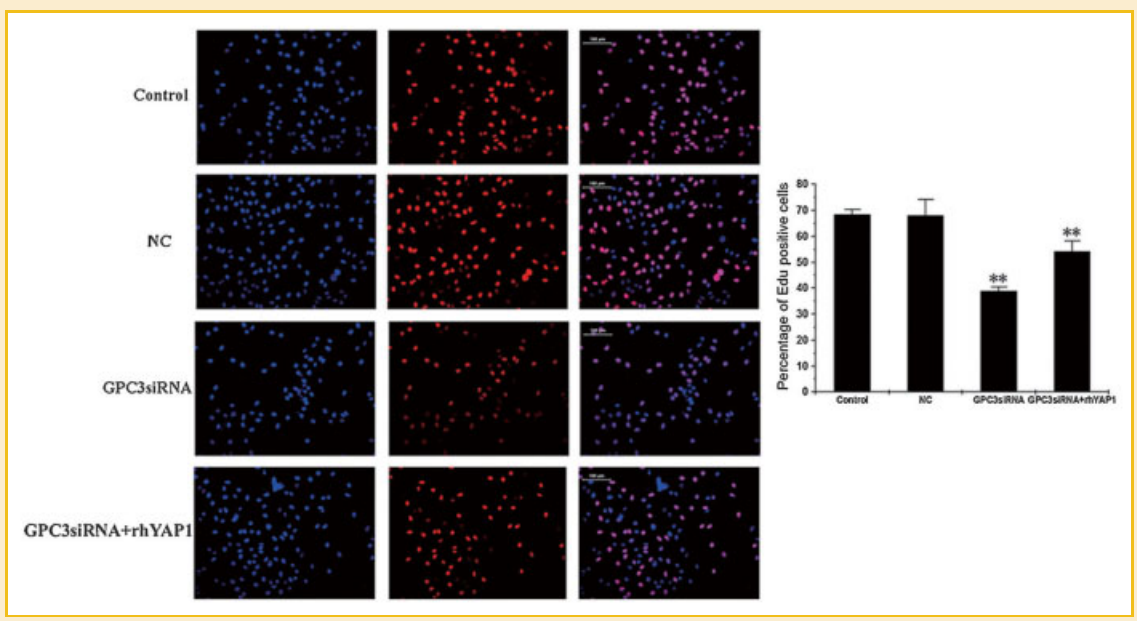

Fig. 4. Effects of GPC3 and YAP on the proliferation of Huh7 cells. The data represent three independent experiments. The results of EdU incorporation assay were analyzed by confocal microscopy (scale bar, 100 μM) in Huh7 cells infected with GPC3 siRNA or negative control siRNA. The EdU incorporation rate was expressed as the ratio of EdU-positive cells to the total DAPI-positive cells. Each bar represents the mean ± SD from three samples ($^{**}P < 0.01$ vs. the control).

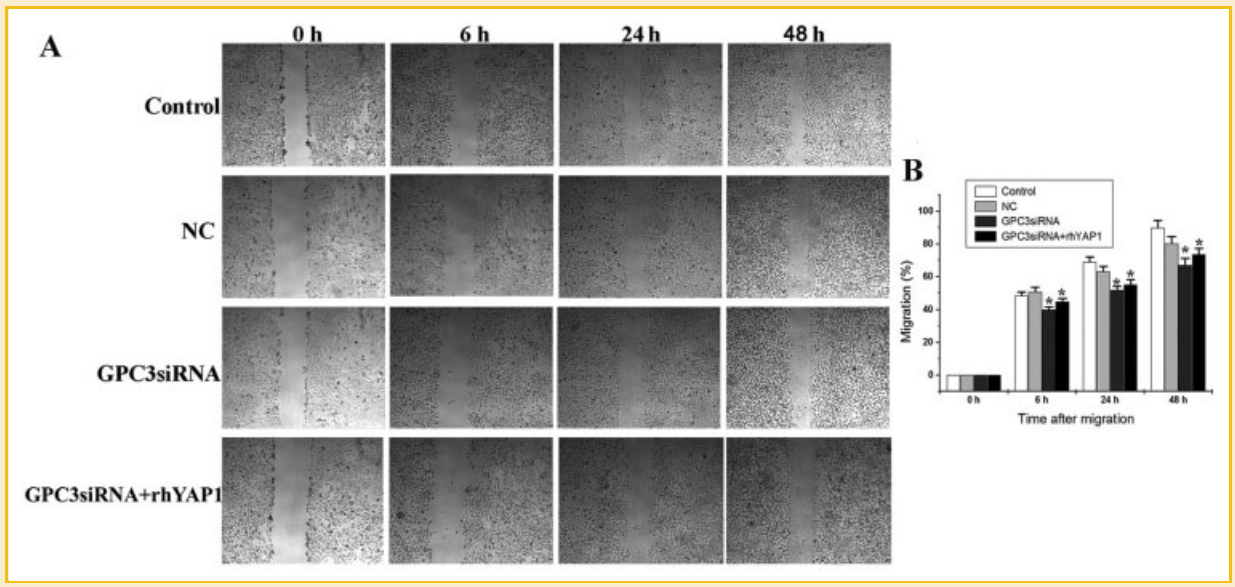

Fig. 5. A: GPC3 gene knockdown reduces HCC cell migration in vitro. B: Statistical analysis. Each bar represents the mean ± SD from three samples ($^{*}P < 0.01$ vs. the control).

proliferation, migration, and invasion, through induction of apoptosis. These results were consistent with those of Ruan et al. [2011], which showed that MHCC97-H cell proliferation and invasion were completely inhibited by knockdown of GPC3 using GPC3-targeted shRNA. These results suggest that *GPC3* could be an oncogene that plays an important role in HCC development. Capurro et al. [2005] also found that GPC3 promoted HCC cell proliferation by increasing the expression of signaling molecules of classical Wnt pathway. Furthermore, both in vitro and in vivo studies have demonstrated that Wnt pathway and Wnt-pathway-dependent cancer cell growth were effectively inhibited by mutant GPC3 that lacked the membrane anchor domain GPI. Kittaka et al. [2008] also confirmed that GPC3 is an important protein in activating the integrin pathway, and may promote proliferation and invasion of HCC cells. IGF signaling pathway was also found involved in GPC3-mediated oncogenesis [Cheng et al., 2008]. Lai et al. [2008, 2010] demonstrated that sulfatase 2 upregulated GPC3 expression and promoted HCC cell proliferation via FGF and Wnt signaling pathway. Sun et al. [2011] found that GPC3 transfection effectively inhibited the cell proliferation through induction of G0/G1 cell cycle arrest. In contrast to the above studies, Kwack et al. [2006] reported that GPC3 could inhibit FGF-2-mediated HCC cell proliferation, migration, and invasion. This study also demonstrated that blocking endogenous GPC3 expression promoted the growth of HCC cells, whereas the forced expression of GPC3-reduced SNU423 cell growth. Liu et al. [2010b] have found that overexpression of GPC3 suppressed hepatocyte proliferation and liver regeneration in GPC3 transgenic mice. Collectively, the contradictory of these findings may be explained by the diversity of different HCC cell lines.

Studies have demonstrated the crosstalk of GPC3 with other signaling pathways. However, its interaction with Hippo pathway has not been reported. Recently, YAP has been identified as an oncogene, and new progress in establishing its role in liver cancer has been clarified. For instance, Zender et al. [2006] used genomics and transcriptomics techniques to identify and validate YAP and cIAP1 as oncogenes in liver cancer. Li et al. [2012] also found the increased YAP expression in HCC cells. However, downregulation of YAP using siRNA resulted in decreased HCC cell survival. Liu et al. [2010b] reported a series of changes in the gene expression profile of GPC3-transgenic mice by gene array analysis, including a 1.4-fold downregulation of YAP. Using quantitative RT-PCR, Lu et al. [2010] observed the GPC3 upregulation by the liver-specific overexpression of a constitutively active mutant YAP protein. Therefore, a underlying link between GPC3 and YAP should be existed in the development of HCC. In the present study, we displayed that GPC3 gene knockdown triggered the downregulation of YAP in HCC cells. However, rhYAP protein effectively blocked the apoptosis induced by GPC3 knockdown. Taken together, our results suggest that GPC3 may modulate the Hippo pathway via YAP, thus affecting the HCC development.

In conclusion, our study reveals that GPC3 promotes the growth of HCC by increasing cell proliferation, enhancing metastasis as well as invasion. Furthermore, GPC3 may collaborate with YAP to regulate the HCC development.

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