Glypican-3 Promotes the Growth of Hepatocellular Carcinoma by Stimulating Canonical Wnt Signaling

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Abstract

Glypican-3 (GPC3) is a heparan sulfate proteoglycan that is bound to the cell membrane by a glycosyl-phosphatidylinositol anchor. GPC3 is expressed by most hepatocellular carcinomas but not by normal hepatocytes and benign liver lesions. We report here that GPC3 stimulates the *in vitro* and *in vivo* growth of hepatocellular carcinoma cells by increasing autocrine/paracrine canonical Wnt signaling. Coimmunoprecipitation experiments showed that GPC3 is able to form complexes with Wnts, and cell-binding assays indicated that GPC3-expressing cells have an increased capacity to bind Wnt. Collectively, these results suggest that GPC3 stimulates Wnt activity by facilitating the interaction of this polypeptide with its signaling receptors. Surprisingly, in contrast to the current model that proposes that Wnt-glypican binding is mediated by the heparan sulfate chains, we found that the nonglycanated GPC3 core protein can form complexes with Wnts. Furthermore, we showed that the glycosaminoglycan chains are not required for the stimulatory effect on Wnt signaling and hepatocellular carcinoma growth. (Cancer Res 2005; 65(14): 6245-54)

Introduction

Hepatocellular carcinoma is one of the most common cancers in the world, with an estimated incidence of 1,000,000 cases per year (1). In some parts of the developing world, it is the most common cause of death from cancer. Although the incidence of hepatocellular carcinoma is lower in the developed Western world, the number of cases is increasing. In particular, it has been predicted that the burden of hepatocellular carcinoma in the United States will augment drastically in 20 to 30 years (2). This predicted higher incidence has been associated with a projected 4-fold increase in the prevalence of chronic hepatitis C infection in the next decade (3).

The study of the pathogenesis of hepatocellular carcinoma has shown that the molecular basis of the malignant phenotype is highly heterogeneous, and it is thought that several of the proteins that play a role in the progression of this disease remain to be identified (4–6). As a result of a search for genes that could play a role in hepatocellular carcinoma, Hsu et al. reported that glypican-3 (GPC3) mRNA levels are significantly elevated in most hepatocellular carcinomas compared with normal liver and nonmalignant liver lesions (7). This result was later confirmed by another group (8). Recently, our laboratory generated monoclonal antibodies (mAb) against GPC3 and used these antibodies to show that the up-regulation of GPC3 in hepatocellular carcinoma can also be observed at the protein level (9). By staining fixed tissue sections, we showed that 21 of 29 (72%) hepatocellular carcinoma sections display GPC3 expression, whereas this protein was undetectable in hepatocytes from normal liver and benign liver diseases. Moreover, by using an ELISA, we found that whereas GPC3 is undetectable in the serum of healthy donors and patients with hepatitis, its levels were significantly increased in 18 of 34 patients (53%) with hepatocellular carcinoma. Similar results have also been reported independently by two other groups (10, 11). Based on these results, it has been proposed that GPC3 could be used as a serum and histochemical marker for hepatocellular carcinoma (9, 10).

Glypicans are a family of heparan sulfate proteoglycans that are linked to the exocytoplasmic surface of the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor (12–15). To date, six glypicans have been identified in mammals (GPC1 to GPC6; refs. 15, 16). In general, glypicans are expressed predominantly during development. Their expression levels change in a stage- and tissue-specific manner, suggesting that they are involved in morphogenesis (15). Although the degree of amino acid homology between most glypicans is moderate, the location of 14 cysteine residues is conserved, suggesting the existence of a highly similar three-dimensional structure (17). Another shared feature of glypicans is the location of the heparan sulfate insertion sites, which seems restricted to the last 55 amino acids in the COOH terminus, placing the heparan sulfate chains close to the cell membrane (15).

Genetic and functional studies done in *Drosophila, Xenopus*, zebrafish, and mammals have shown that glypicans are required for the optimal activity of Wnts, Hedgehogs, and bone morphogenetic proteins in specific tissues (18–27). In the case of Wnts, glypicans have been reported to stimulate both the canonical and noncanonical pathways (21, 22, 25). Because Wnts are known to bind to heparan sulfate (28), it has been proposed that the stimulatory activity of glypicans is based on their ability to act as facilitators of the interaction between Wnts and their receptors (18). This hypothesis has been supported by the finding that several Wnts can coimmunoprecipitate with glypicans (22, 23, 29).

Although the molecular pathogenesis of hepatocellular carcinoma is highly heterogeneous (4, 30–32), activation of the canonical Wnt signaling pathway has been found to be one of the most frequent events associated with malignant transformation of liver cells (30, 31, 33) Specifically, activating mutations in β-catenin have been reported in ~18% of hepatocellular carcinoma patients and axin mutations in ~6% (34). Furthermore, overexpression of Frizzled-7 in a large proportion of hepatocellular carcinomas has been recently reported (35). Based on the capacity of glypicans to increase Wnt signaling, we have hypothesized that overexpressed GPC3 promotes hepatocellular carcinoma growth by stimulating the canonical Wnt pathway. Here we provide experimental evidence supporting such hypothesis. In addition, we report that the heparan sulfate chains of GPC3 are not required for its growth-stimulatory activity in hepatocellular carcinoma.

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Materials and Methods

Cell lines and plasmids. PLC-PRF-5, HLF, and HepG2 liver cancer cell lines were cultured in MEM with 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA) supplemented with MEM nonessential amino acid solution (Life Technologies, Gaithersburg, MD) and sodium pyruvate (1 mmol/L). The 293 and 293T cell lines were cultured in DMEM and 10% FBS. The PLC-PRF-5, HepG2, and 293 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and Dr. Eiji Miyoshi (University of Osaka) donated HLF cell line. L cells permanently transfected with Wnt3A-pLNCx or empty vector (pLNCx) as control were obtained from ATCC, and cultured in DMEM containing 10% FBS. To collect conditioned medium, the cells were grown at high density for 4 days. Rat1 cells permanently transfected with Wnt1-pLNCx or empty vector (pLNCx) were obtained from Dr. J. Kitajewski (Columbia University), and cultured in DMEM 10% FBS. Mouse DKK1 was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) and transiently transfected into the 293T cell line to generate conditioned medium, which was collected 48 hours after transfection.

Expression vectors containing Hemagglutinin A–tagged GPC3, the mutant GPC3 that cannot be glycanated (GPC3ΔGAG) and the mutant GPC3 lacking the GPI-anchoring domain (GPC3ΔGPI), were previously described (36). Hemagglutinin A–tagged Wnt1, Wnt3, Wnt3A, and Wnt7B expression vectors were obtained from Dr. J. Kitajewski and the Wnt2b cDNA was provided by Dr. Vainio (University of Oulo).

Generation of stable GPC3-expressing hepatocellular carcinoma cell lines. The PLC-PRF-5 and HLF cell lines were transfected with a selectable expression vector containing the full-length wild-type GPC3 cDNA (GPC3), the GPC3ΔGAG cDNA, the GPC3ΔGPI cDNA, or with vector alone (EF) as a negative control. The transfections were done by using Lipofectin according to the manufacturer's instructions. After selection and expansion, cells were stained with the anti-GPC3 1G12 mAb and a FITC-conjugated secondary antibody, and cells expressing high levels of GPC3 were isolated by fluorescence-activated cell sorting (FACS).

Cell proliferation assay. Cells were plated into 24-well dishes (20,000 cells per well) in medium without antibiotics. At the indicated time points cells were trypsinized and counted with a hematocytometer. Each experiment was done at least four times by quadruplicates. When indicated, the proliferation assay was done in the presence of DKK1 or control (pcDNA) conditioned medium diluted with an equal amount of regular medium. The conditioned media were replaced with fresh conditioned medium every 24 hours during the course of the experiment.

Western blotting. To assess GPC3 expression, cells were lysed in radioimmunoprecipitation assay buffer and the lysates analyzed by Western blot using anti-GPC3 mAb as previously described (9). Membranes were then reprobed with the anti-actin AC-40 mAb (Sigma, St. Louis, MO) as a loading control. The presence of DKK1 in the conditioned medium was detected with anti-DKK1 goat polyclonal antibody (AF1765, R&D Systems, Minneapolis, MN).

Tumorigenicity assay. Cells (5×10^6) were injected s.c. into the left flank of 7-week-old CB-17 SCID mice (Charles River, Wilmington, MA) using a 26-gauge needle. At the indicated time points, the volumes of the tumors were determined by measuring the largest (a) and smallest (b) axis using a caliper. Volume was calculated according to the formula $V = 0.5ab^2$. The experiment was done twice with similar results. Significance of differences between the groups of mice was determined by the Mann-Whitney test, and the level of significance was set as $P < 0.05$. Mice handling and experimental procedures were done in accordance with institutional guidelines.

Cytoplasmic β-catenin determination. Cells were grown in the absence of serum for 48 hours, washed extensively with PBS, and harvested with a cell scrapper. Cells were then resuspended in TBS [10 mmol/L Tris-HCl (pH 7.4), 140 mmol/L NaCl, containing 2 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, and 1 μg/mL leupeptin], and homogenized by 10 strokes in a Dounce homogenizer. After low speed centrifugation (3,000 rpm 10 minutes at 4°C), the crude supernatant was submitted to ultracentrifugation in a Beckman SW60 rotor at 27,000 rpm for 90 minutes at 4°C to obtain the soluble cytoplasmic fraction and a membrane-rich pellet. The level of cytoplasmic β-catenin was assessed by Western blot with the antibody clone 14 (BD Transduction Laboratories, San Jose, CA) We used actin or CDK4 (antibody C-22, Santa Cruz Biotechnology, Santa Cruz, CA) as loading controls.

RNA isolation and reverse transcription-PCR analysis. Total RNA was prepared using Trizol (Invitrogen) according to manufacturer's instructions. Reverse transcription was carried out with SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen). As a control, duplicate cDNA synthesis reactions were done for each experiment without the addition of reverse transcriptase. Different pairs of gene-specific primers (37–51) were used for PCR analysis, and in all cases 30 cycles of amplification were done.

Effect of GPC3 on the transcriptional activity induced by exogenous Wnt. Cells were plated in a 24-well plate at a density of 70,000 cells per well and cotransfected with a luciferase reporter vector driven by the TOPFLASH promoter, which contains several TCF/β-catenin–responsive elements (52), and a β-galactosidase expression vector using Lipofectin (Invitrogen). Twenty-four hours after transfection, the cells were incubated for 6 hours with conditioned medium from Wnt3A-transfected L cells or cells transfected with vector control. Alternatively, transfected cells were cocultured with 300,000 Wnt1-expressing Rat1 cells or with 293 cells transiently transfected with Wnt1, Wnt3A, Wnt2B, Wnt3, and Wnt7B for 16 hours. Cells were then lysed and luciferase activity measured according to the instructions of the Luciferase Assay System (Promega, Madison, WI). Ten microliters of lysates were used for β-galactosidase activity determination, and each luciferase value was normalized for transfection efficiency using such activity. Each experiment was done at least four times by triplicates.

Wnt3A iodination and binding assay. Two micrograms of recombinant mouse Wnt3A (R&D Systems) were iodinated with 1 mCi of Iodine-125 (Amersham Biosciences, Little Chalfont, United Kingdom) by the iodogen method using iodogen-precoated reaction tubes (Pierce, Rockford, IL) according to manufacturer's instructions. For the binding assay, stably transfected PLC-PRF-5 cells were plated in a 48-well plates at a density of 100,000 cells per well. Cells were allowed to attach overnight and washed twice with ice-cold medium. A binding solution containing different concentrations of ^{125}I-Wnt3A (range, 2.5-160 ng/mL) in ice-cold serum-free medium was added for 3 hours at 4°C. The binding solution was then removed and the cells were gently washed thrice with ice-cold serum-free medium. The cells were lysed in 0.2 N NaOH for 15 minutes, and the extracts were counted in a gamma counter. The experiment was done thrice by triplicates.

Coimmunoprecipitation. 293T cells were transfected with GPC3 and Hemagglutinin A–tagged Wnt expression vectors using LipofectAMINE 2000 (Invitrogen). Two days after transfection, cell lysates were prepared in 1% Triton X-100, 0.5% sodium deoxycholate in PBS. Lysates containing 400 μg of proteins were precleared with protein G Sepharose (fast flow, Sigma) during 1 hour at 4°C. After centrifugation, GPC3 was immunoprecipitated from the precleared lysates adding anti-GPC3 1G12 mAb (2.5 μg/mL) overnight at 4°C. After washing, the beads were collected and the presence of Wnt in the immunoprecipitated material analyzed by Western blot using an anti-Hemagglutinin A antibody (3F10, Boehringer Mannheim, Indianapolis, IN). To control for efficiency of transfection, GPC3 and Wnt levels were assessed by Western blot.

Western blot analysis of organs from GPC3-transgenic mice. Organs were obtained from 3-week-old mice, weighed, and immediately frozen in dry ice. Frozen tissues were chopped in small pieces, dispersed in TBS containing 50 mmol/L sodium fluoride and 5 mmol/L sodium vanadate, and homogenized by 40 to 50 strokes in a Dounce homogenizer. For total lysate preparation, part of the homogenate was diluted with 1 volume of 2× lysis buffer (2% Triton X-100, 0.2% SDS, 2 mmol/L EDTA, in TBS) and incubated for 30 minutes on ice. To obtain cytoplasmic lysates, the cell homogenate was first centrifuged at 3,000 rpm for 10 minutes at 4°C, and the crude supernatant was then ultracentrifuged in a Beckman SW60 rotor at 27,000 rpm for 90 minutes at 4°C. The levels of GPC3 and cytoplasmic β-catenin were assessed by Western blot as described above. Each experiment was repeated with three independent pairs of mice belonging to three independent litters. The bands obtained from the Western blot analysis were scanned and analyzed by densitometry (Quantity-1).

Results

GPC3 stimulates *in vitro* and *in vivo* growth of hepatocellular carcinoma cells. To test the hypothesis that GPC3 plays a role in hepatocellular carcinoma growth, we transfected a GPC3 expression vector into the PLC-PRF-5 and HLF hepatocellular carcinoma cell lines, which express very little and no GPC3, respectively. After antibiotic selection, transfected cells were pooled and expanded, and a population of hepatocellular carcinoma cells expressing high levels of GPC3 was sorted from the transfected cells with a FACS. As shown in Fig. 1*A* and *B*, the FACS-selected cells (GPC3) display higher levels of GPC3 expression than the original presorted population of GPC3-transfected cells (psGPC3). It is important to note, however, that the levels of ectopic GPC3 produced by the transfected hepatocellular carcinoma cell lines are within the physiologic range, because they are lower than the endogenous levels expressed by HepG2, a GPC3-positive hepatocellular carcinoma cell line (Fig. 1*C*). Next, the effect of ectopic GPC3 on the proliferation rate of the transfected cells was investigated. It was found that GPC3 significantly stimulates the proliferation rate of the two transfected hepatocellular carcinoma cell lines in a dose-dependent manner (Fig. 1*A* and *B*). No cell death was observed at any time point during the proliferation assay. Similar results were obtained when the growth curves were done in serum-free conditions (data not shown). Because glypicans are known to stimulate the activity of various growth factors (23, 53, 54), these results suggest that GPC3 is stimulating the autocrine/paracrine activity of one or more growth factors secreted by the hepatocellular carcinoma cell lines.

Although glypicans have been shown to stimulate the activity of several "heparin-binding" growth factors through their heparan sulfate chains, in some cellular systems mutant glypicans that cannot be glycanated conserve at least part of the functional properties of the wild-type counterparts (22, 23, 36). We decided, therefore, to investigate whether glycanation is required for the stimulatory effect of GPC3 on the proliferation of hepatocellular carcinoma cells. To this end, the PLC-PRF-5 and HLF cell lines were transfected with a mutated GPC3 that cannot be glycanated (GPC3ΔGAG; ref. 36). After transfection, GPC3ΔGAG-expressing cells were sorted by FACS and expanded. GPC3ΔGAG expression levels where then probed by Western blot (Fig. 1*D*). Assessment of the proliferation rate of the GPC3ΔGAG-transfected cells showed that in PLC-PRF5 cells this GPC3 mutant was also able to stimulate cell proliferation, albeit at a lower level than wild-type GPC3. This reduced effect on cell proliferation by the mutant GPC3 was not due to lower expression levels because both wild-type and mutant GPC3-transfected cells showed similar levels of expression when analyzed by FACS (data not shown). We conclude, therefore, that although the heparan sulfate chains are required for optimal *in vitro* stimulation of the PLC-PRF-5 cells, the GPC3 protein core has stimulatory activity on its own. In contrast, GPC3ΔGAG had no effect on the proliferation rate of the HLF cells (Fig. 1*D*).

Because the *in vivo* proliferation of tumor cells is influenced by factors that do not play a role *in vitro*, we investigated whether

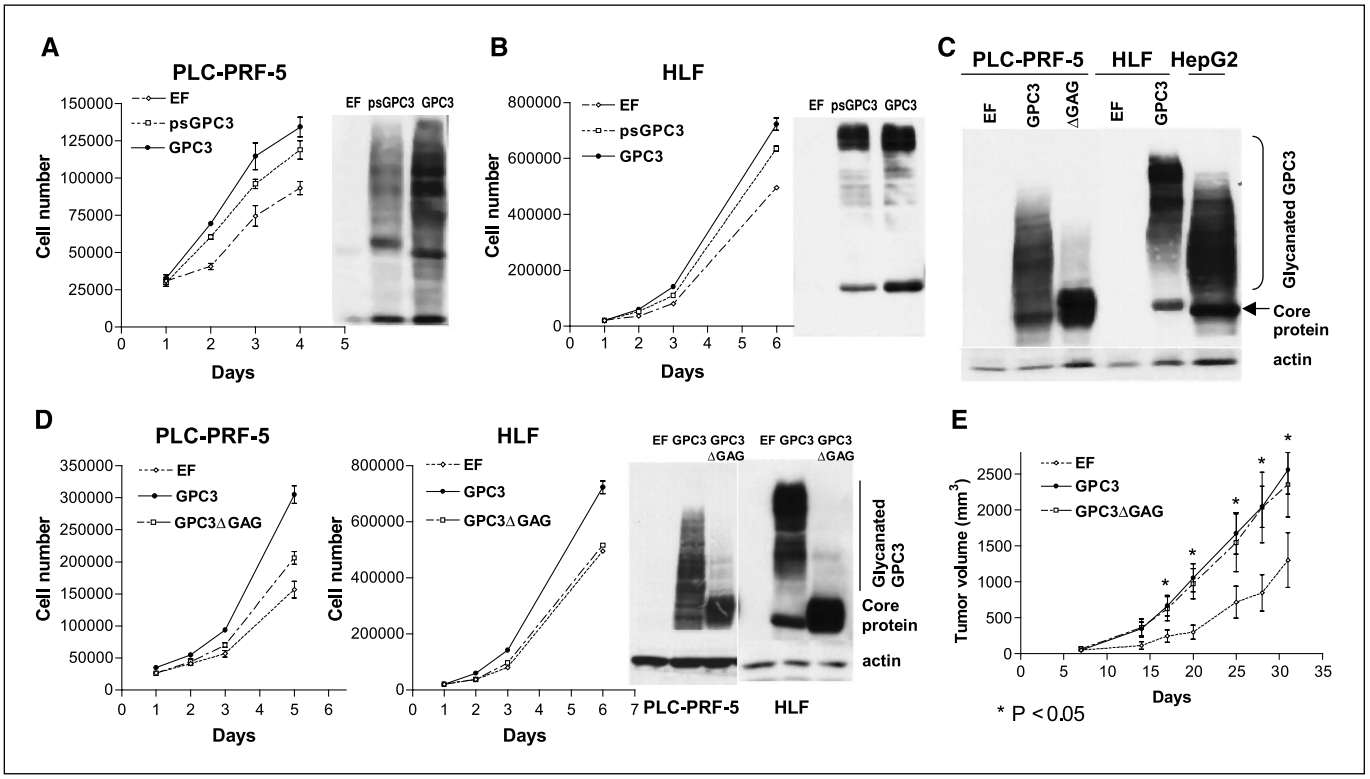

Figure 1. GPC3 stimulates *in vitro* and *in vivo* growth of hepatocellular carcinoma cells. Proliferation rate of FACS-selected PLC-PRF-5 (*A* and *D*) and HLF (*B* and *D*) cell lines transfected with expression vectors containing wild-type GPC3 (GPC3), a mutant GPC3 that cannot be glycanated (GPC3ΔGAG), and vector alone (EF). The proliferation rates of the presorted population of cells (psGPC3) are also shown (*A* and *B*). Cells were counted at the indicated time points. *Points,* averages of quadruplicates; *bars,* ±SD. Representative experiment of four. Levels of GPC3 expression (*right*). *C,* Western blot analysis of GPC3 levels in GPC3-transfected cells and in the GPC3-positive HepG2 cells. Actin was used as a loading control. *E, in vivo* growth of PLC-PRF-5 cells transfected with expression vectors containing GPC3, GPC3ΔGAG, and vector alone (EF). *Points,* average volumes of tumors from 10 mice/group; *bars,* ± SD. *, difference between GPC3 and GPC3ΔGAG and EF control group is statistically significant. The experiment was done twice with similar results; representative experiment.

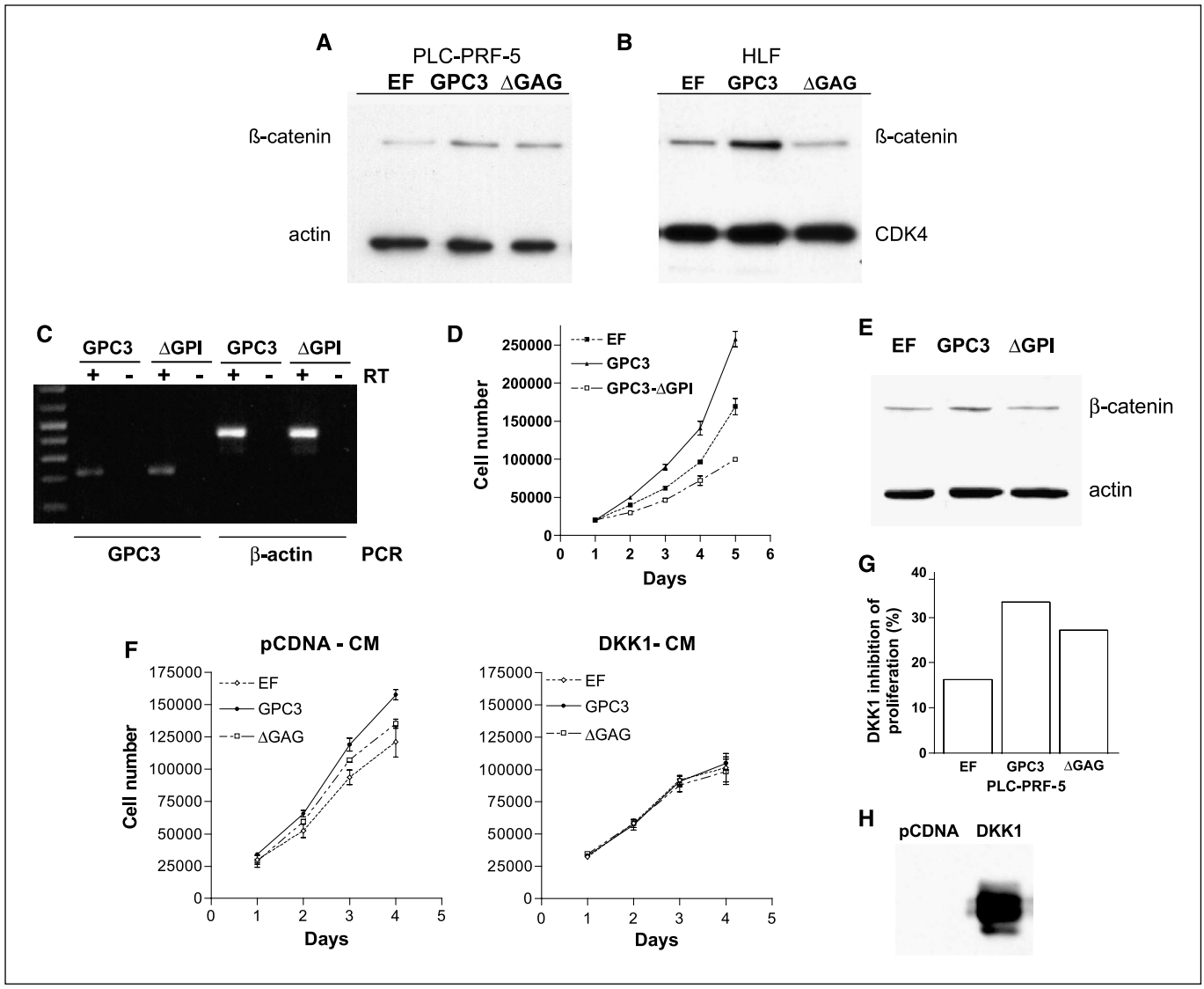

Figure 2. GPC3 promotes hepatocellular carcinoma growth by stimulating the canonical Wnt pathway (*A-B*) GPC3 induces the stabilization of cytoplasmic β-catenin. Western blot analysis of cytoplasmic β-catenin in PLC-PRF-5 (*A*) or HLF (*B*) cells transfected with GPC3, GPC3ΔGAG (ΔGAG), or vector alone (EF). Actin or CDK4 were used as loading controls. One representative result of three. *C-E,* soluble GPC3 lacks stimulatory activity. PLC-PRF-5 cells were transfected with expression vectors containing wild-type GPC3 (GPC3) or a mutant GPC3 lacking the GPI-anchoring domain (GPC3ΔGPI) and vector alone (EF). *C,* total RNA was isolated from these cells and cDNA was synthesized using reverse transcriptase (*RT*, +). As a negative control, the same RNAs were incubated in the absence of reverse transcriptase (−). PCR was then done using GPC3 or β-actin specific primers. *D,* proliferation rate of the transfected cells was analyzed and the number of cells counted by quadruplicates at the indicated time points. Representative experiments of three. *E,* Western blot analysis of cytoplasmic levels of β-catenin in the indicated transfected cells using actin as loading control. One representative result from three independent experiments. *F-H,* DKK1 inhibits GPC3-induced proliferation. *F,* proliferation rate of PLC-PRF-5 [EF, GPC3, or ΔGAG-GPC3 (ΔGAG)] was assessed in the presence of conditioned medium from 293T cells transfected with DKK1 (DKK1-CM) or vector control (pCDNA-CM). Cell counts were done by quadruplicates at the indicated time points. The degree of DKK1-induced inhibition of cell proliferation compared with control conditioned medium (as percentage) is indicated in (*G*). *H,* Western blot analysis shows the presence of DKK1 in the conditioned medium.

GPC3 also stimulates the tumorigenicity of hepatocellular carcinoma cells growing as xenografts in mice. To this end, we used the PLC-PRF-5 cell line, which has been reported to be tumorigenic in mice (55). As shown in Fig. 1*E*, we found that cells transfected with GPC3 or nonglycanated GPC3 grow significantly faster in SCID mice compared with vector-transfected cells. It is important to note, however, that unlike the results obtained in tissue culture, the degree of growth stimulation induced by nonglycanated GPC3 was similar to that induced by wild-type GPC3.

GPC3 induces the stabilization of β-catenin in hepatocellular carcinoma cells. As discussed above, the canonical Wnt signaling pathway is frequently activated in hepatocellular carcinoma. Because glypicans have been shown to increase Wnt activity in several cell types (21, 23) and canonical Wnt signaling is known to stimulate cell proliferation (56, 57), we decided to investigate the effect of GPC3 overexpression on canonical Wnt signaling in hepatocellular carcinoma cells. To this end, we studied the effect of GPC3 on the levels of cytoplasmic β-catenin in the PLC-PRF-5 and HLF cell lines. As shown in Fig. 2*A* and *B*, we found that both GPC3-transfected cell lines display a significant accumulation of cytoplasmic β-catenin. We conclude, therefore, that GPC3 stimulates Wnt signaling in hepatocellular carcinoma cells. Consistent with the fact that nonglycanated GPC3 is still able to stimulate the proliferation of PLC-PRF-5 cells (Fig. 1*D*), we

found that the Wnt canonical pathway is also activated by the GPC3ΔGAG mutant in such cells (Fig. 2*A*). On the other hand, the GPC3ΔGAG mutant did not induce the accumulation of β-catenin in the HLF cells (Fig. 2*B*). This is consistent with the lack of effect of this mutant on the proliferation of HLF cells (Fig. 1*D*).

Attachment to the cell surface is required for the stimulatory activity of GPC3. If, as proposed by other investigators (18), glypicans stimulate Wnt activity by facilitating their interaction with the signaling receptors, a mutant GPC3 that cannot be attached to cell surface should not be able to stimulate cell proliferation and Wnt signaling in hepatocellular carcinoma cells. To test this hypothesis, PLC-PRF-5 cells were transfected with a mutated GPC3 lacking the GPI-anchoring domain (GPC3ΔGPI; ref. 36). GPC3ΔGPI-expressing cells were sorted by FACS, and the level of ectopic GPC3ΔGPI expression was assessed by RT-PCR (Fig. 2*C*). As shown in Fig. 2*D*, GPC3ΔGPI did not stimulate the proliferation of PLC-PRF-5 cells, indicating that GPC3 has to be bound to the cell surface to be able to stimulate cell proliferation. Consistent with this observation, GPC3ΔGPI did not increase the levels of cytoplasmic β-catenin (Fig. 2*E*). In fact, the proliferation rate GPC3ΔGPI-transfected cells was slower than that of the vector-transfected cells, suggesting that the GPC3 released into the medium is probably competing for the binding of one or more promitogenic peptide. Because the levels of cytoplasmic β-catenin are not reduced in the GPC3ΔGPI-transfected cells compared with vector control cells and endogenous Wnts are not a limiting factor in the canonical pathway of PLC-PRF-5 cells (as shown in Fig. 1), we propose that the secreted GPC3 is inhibiting cell growth by competing for a growth factor that is not a Wnt.

To verify that the GPC3-induced increase in cell proliferation rate is due to the ability of this glypican to stimulate Wnt signaling, we used the Wnt antagonist DKK1. DKK1 specifically inhibits canonical Wnt signaling at the level of ligand-receptor interaction by binding the Wnt coreceptor LRP6 and inducing its endocytosis (58). To test the effect of DKK1 in the cell proliferation assay, we added conditioned medium obtained from DKK1- or vector control (pcDNA)–transfected 293T cells (Fig. 2*H*). If GPC3 stimulates cell proliferation in a Wnt-dependent manner, DKK should abolish such stimulatory activity. Figure 2*F* shows that this is indeed the case: in the presence of DKK1, neither GPC3 nor GPC3ΔGAG was able to stimulate the proliferation of PLC-PRF-5 cells. DKK1 was also able to inhibit the proliferation of EF-PLC-PRF-5 cells, suggesting that these cells are able to respond to Wnt even in the absence of GPC3 (Fig. 2*F* and *G*).

Expression of Wnt family genes in hepatocellular carcinoma cells. Hepatocellular carcinomas are known to express various Wnt family members (59). Because both the GPC3-induced stimulation of cell proliferation and the GPC3-induced accumulation of cytoplasmic β-catenin in hepatocellular carcinoma cells occur even in serum-free conditions, we hypothesized that GPC3 is increasing the autocrine/paracrine activity of one or more Wnts produced by the hepatocellular carcinoma cells. As a first step

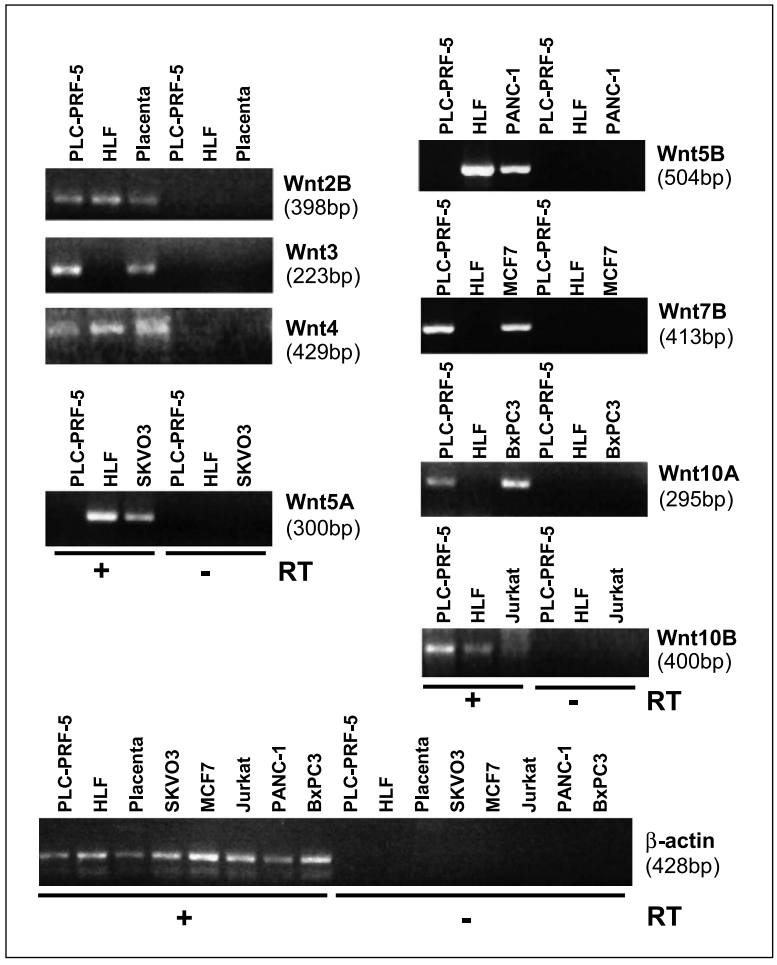

Figure 3. RT-PCR analysis of Wnt genes in PLC-PRF-5 and HLF cell lines. Total RNA was isolated from PLC-PRF-5, HLF, MCF-7, SKVO3, PANC-1, BxPC3, and Jurkat cell lines or human placenta, and cDNA was synthesized using reverse transcriptase (*RT*, +). As a negative control, the same RNAs were incubated in the absence of reverse transcriptase (−). PCR was then done with specific primers. Only includes results for the Wnts in which at least one of the cell lines was positive [gene name (*right*) along with the size of the amplicon]. The positive controls used were human placenta for Wnt2B, Wnt3, and Wnt4; SKVO3 cells for Wnt5A; PANC-1 cells for Wnt5B; MCF-7 cells for Wnt7B; BxPC3 cells for Wnt10A and Jurkat cells for Wnt10B. Human β-actin was used as a constitutively expressed marker to assess relative amounts of RNA in each sample.

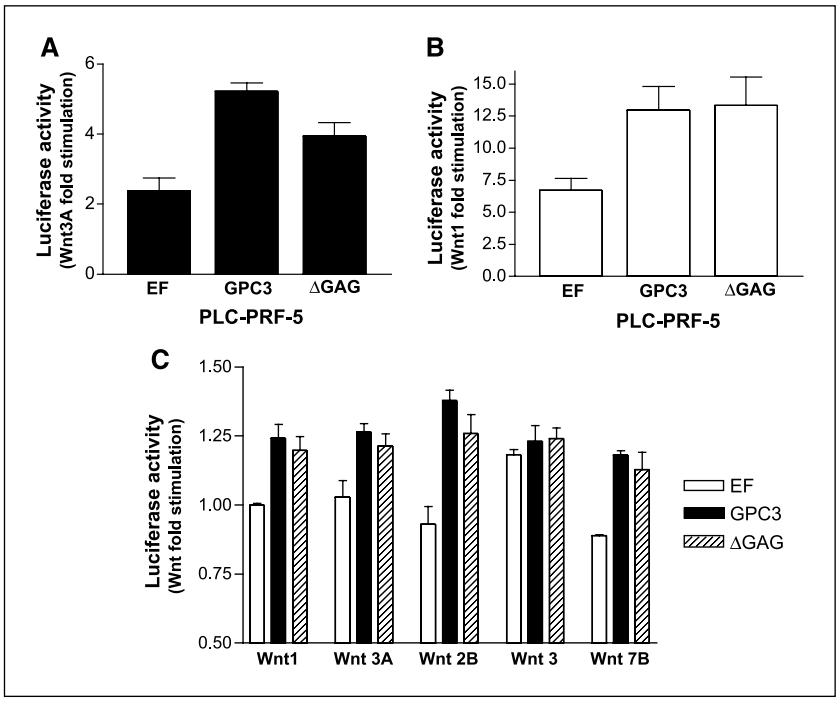

Figure 4. Effect of GPC3 on the transcriptional activity of β-catenin induced by exogenous Wnt. Luciferase activity of transfected PLC-PRF-5 cells [EF, GPC3, or GPC3ΔGAG (ΔGAG)] in response to: Wnt3A-transfected L cells conditioned medium (*A*); coculture with Wnt1-expressing Rat1 cells (*B*); or coculture with 293 cells transiently transfected with Wnt1, Wnt3A, Wnt2B, Wnt3, and Wnt7B (*C*). Luciferase activity was normalized for transfection efficiency using β-galactosidase activity. The experiments were done four times by triplicates. *Columns,* fold stimulation induced by Wnt on the normalized luciferase activity (average of the four different experiments); *bars,* ±SD.

towards the verification of this hypothesis, we investigated the expression of all Wnt family members in the PLC-PRF-5 and HLF cell lines. To this end, we did RT-PCR using specific primers. As shown in Fig. 3, PLC-PRF-5 cells express Wnt2B, Wnt3, Wnt4, Wnt7B, Wnt10A, and Wnt2B and HLF cells express Wnt2B, Wnt4, Wnt5A, and Wnt5B. These results are consistent with the hypothesis that GPC3 is stimulating Wnt autocrine/paracrine activity.

GPC3 increases the response to exogenous canonical Wnts in PLC-PRF-5 cells. In an *in vivo* environment, Wnts can be produced not only by the hepatocellular carcinoma cells but also by various cell types in the tumor stroma. We decided therefore to investigate whether GPC3 can also increase the response to exogenous Wnts. To this end, we assessed the effect of GPC3 on Wnt-induced transcriptional activity, using the TOPFLASH-luciferase reporter assay (52). To provide exogenous Wnts to the reporter-transfected cells, we added conditioned medium obtained from Wnt3A-transfected L cells, or we cocultured them with Wnt1-transfected Rat1 cells. These two sources of Wnt have been extensively used for the reporter assays, and hepatocytes are able to respond to them (60, 61). As shown in Fig. 4*A* and *B*, GPC3 significantly stimulated the response of PLC-PRF-5 cells to both Wnt3A and Wnt1. Interestingly, the luciferase activity generated by GPC3ΔGAG-transfected cells in response to Wnt3A conditioned medium was lower than that of GPC3 wild type–transfected cells (Fig. 4*A*). On the other hand, both wild-type and mutant GPC3 induced a similar increase in the response to Wnt1 produced by the cocultured cells (Fig. 4*B*).

We next assessed the effect of GPC3 on the response of PLC-PRF-5 cells to the Wnts that are expressed by these cells. To provide the various Wnts, we cocultured the PLC-PRF-5 cells with 293 cells transiently transfected with Wnt2B, Wnt3, and Wnt7B. We found that GPC3 and GPC3ΔGAG significantly increase the response of PCL-PRF-5 to exogenous Wnt2B and Wnt7B, whereas there was no effect on the response to Wnt3 (Fig. 4*C*). It should be noted that the degree of signaling stimulation induced by GPC3 on exogenous Wnts produced by cocultured transiently transfected 293 cells was lower than that induced on Wnts produced by L and Rat1 cells. Because this lower level of stimulation was also seen with Wnt3A and Wnt1 produced by transiently transfected 293 cells (Fig. 4*C*), we speculate that these lower levels could result from the way in which each of the Wnt-producing cell lines secretes Wnt. It has been recently reported that secreted Wnt can be lipidated and that the degree of lipidation could induce variable levels of aggregation and activity (62).

GPC3 increases the binding of Wnt3A to PLC-PRF-5 cells. As discussed above, the hypothesis that glypicans stimulate Wnt activity by facilitating its interaction with the signaling receptors was based on the fact that Wnts are heparin-binding molecules and on the observation that Wnts and glypicans can coimmunoprecipitate. However, in the PLC-PRF-5 cells, we found that the heparan sulfate chains of GPC3 were not required for its growth-promoting activity and for the stimulation of Wnt signaling. This raises the question as to whether in hepatocellular carcinoma cells GPC3 is acting at the level of ligand-receptor interaction. To investigate this, we first assessed the effect of GPC3 on the ability of Wnt to bind to the cell surface. For the binding assay, we used Wnt3A, because it is the only purified canonical Wnt commercially available. As shown in Fig. 5*A*, GPC3-expressing cells exhibited a significantly higher ^{125}I-Wnt3A binding than the vector control-expressing cells, indicating that the presence of GPC3 is increasing the binding of Wnt3A to the cell surface. Certainly, Wnt3A binding to the PLC-PRF-5 cells was noticeable even in the absence of ectopic GPC3. This is not surprising because as Wnt-responsive cells (Fig. 2*F* and *G*), PLC-PRF-5 must express the signaling Wnt receptors Frizzled and LRP5/6. GPC3ΔGAG also significantly stimulated ^{125}I-Wnt3A binding to PLC-PRF-5 cells but to a lesser degree than that observed for wild-type GPC3. This reduced effect of the mutant GPC3 is

consistent with the cell proliferation and luciferase assay results obtained in response to soluble Wnt3A (Fig. 1*D* and Fig. 4*A*). Overall, these results provide support to the hypothesis that GPC3 and GPC3ΔGAG are stimulating Wnt signaling by acting at the level of ligand-receptor interactions.

Wnt and GPC3 coimmunoprecipitate. The cell binding experiments strongly suggest that the protein core of GPC3 has the ability to interact with Wnt3A. To provide additional experimental support to this hypothesis, we did coimmunoprecipitation experiments. 293T cells were transiently transfected with wild-type GPC3 or the GPC3ΔGAG mutant and Hemagglutinin A–tagged Wnt3A, and GPC3 was immunoprecipitated from the cell lysate using an anti-GPC3 mAb. The immunoprecipitated material was then probed for the presence of Wnt3A using an anti-Hemagglutinin A antibody. Figure 5*B* shows that Wnt3A coimmunoprecipitated with both GPC3 and GPC3ΔGAG. Because Wnt3A is not expressed in PLC-PRF-5 cells and our results suggest that GPC3 is acting on autocrine/paracrine Wnts, we also did coimmunoprecipitation experiments using Wnt7B, which is one of the Wnts produced by the PLC-PRF-5 cells (Fig. 3). As it can be seen in Fig. 5*B*, Wnt7B also coimmunoprecipitated with both GPC3 and GPC3ΔGAG. This is consistent with the hypothesis that GPC3 is stimulating autocrine/paracrine Wnt activity.

Differential effect of ectopic GPC3 on Wnt signaling in the liver. Our findings indicate that GPC3 is promoting hepatocellular carcinoma proliferation by stimulating Wnt canonical signaling in the liver. However, ectopic expression of GPC3 in cell lines derived from several types of tumors (54), including breast cancer (37), ovarian cancer (63), lung cancer (64), and mesotheliomas (65) induces growth inhibition. Furthermore, GPC3 has been found to be down-regulated in such tumor types (54), including breast cancer (37), ovarian cancer (63), lung cancer (64), and mesotheliomas (65), suggesting that the loss of GPC3 expression provide a growth advantage. In addition, mutations in GPC3 are the cause of the Simpsom-Golabi-Behmel syndrome (66), an X-linked disorder characterized by developmental overgrowth and several visceral and skeletal abnormalities (67). This indicates that during development GPC3 also acts predominantly as a negative regulator of cell proliferation and as an inducer of cell death in certain cell types.

It seems, therefore, that GPC3 has tissue-specific functions. To provide additional evidence for such specificity, we have analyzed GPC3-transgenic mice recently generated in our laboratory. These mice are viable but they are smaller than the normal littermates (a more detailed study of these mice will be published elsewhere). In these mice, GPC3 expression is driven by a β-actin promoter. Figure 6*A* shows the expression levels of ectopic GPC3 in various tissues. As expected, given the type of promoter driving GPC3 expression, the levels of GPC3 are particularly high in the heart, but all other tissues analyzed, including the liver, display significant levels of this glypican. To investigate the tissue-specific activity of GPC3, we compared the status of the Wnt pathway by assessing the levels of cytoplasmic β-catenin in the various tissues by Western blot. As shown in Fig. 6*B*, we found that such levels were reduced in the kidney, heart, and stomach of the transgenic mice compared with the normal littermates. On the other hand, no significant difference was observed in the lung (it should be noted that this organ already expresses high levels of endogenous GPC3). Interestingly, however, the levels of β-catenin in the GPC3-transgenic liver were significantly elevated compared with the controls. These results provide further evidence for the tissue-specific activity of GPC3 and generate additional support for a stimulatory role of GPC3 in hepatic canonical Wnt signaling.

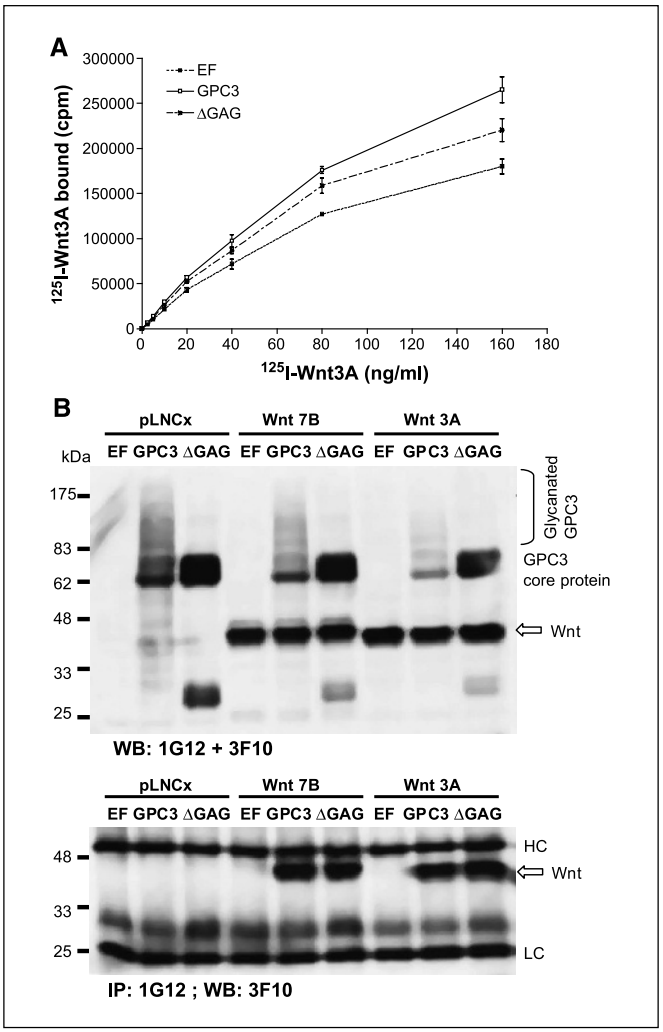

Figure 5. Analysis of Wnt-GPC3 interaction. *A,* binding of ^{125}I-Wnt3A to PLC-PRF-5 cells transfected with GPC3 (GPC3), GPC3ΔGAG (ΔGAG), and vector alone (EF). A representative experiment of three. *Points,* average of triplicates; *bars,* ±SD. *B,* Wnt-GPC3 coimmunoprecipitation. 293T cells were transfected with the indicated expression vectors, and GPC3 was immunoprecipitated with anti-GPC3 mAb (1G12). *Bottom,* the presence of Wnt in the immunoprecipitates was probed with an anti-HA mAb (3F10). *Top,* the amount of ectopic GPC3 and Wnt in the whole lysate of the transfected cells was assessed by Western blot using anti-GPC3 (1G12) and anti-HA (3F10) mAbs. *Right,* position of Wnt (*open arrow*), GPC3 core protein (*arrowhead*), glycanated GPC3 (*brace*), and the IgG heavy chain (*HC*), and IgG light chain (*LC*) from the anti-GPC3 mAb. *Left,* molecular weight marker positions.

Discussion

In this article, we show that GPC3 stimulates the growth of hepatocellular carcinomas. Because this glypican is frequently overexpressed in these tumors, our finding provides a potential novel target for a disease against which there are very limited therapeutic tools (1, 5).

We also show here that GPC3 promotes hepatocellular carcinoma growth by stimulating the canonical Wnt pathway.

The ability of glypicans to activate such pathway has been previously shown, but, to our knowledge, this is the first report demonstrating that glypican-induced activation of Wnt signaling plays a role in disease.

Because GPC3 is able to stimulate the Wnt pathway in a serum-free environment, we propose that, at least in tissue culture conditions, GPC3 is stimulating autocrine/paracrine Wnt signaling. In this regard, it is important to note that Bafico et al. (68) have recently reported that autocrine Wnt activity is increased in many cancers, but the mechanism of this increased Wnt activity was not established. We propose that GPC3 up-regulation could be one of the mechanisms involved in the case of hepatocellular carcinoma.

It is important to note that in addition to Wnts, glypicans have been reported to be able to increase the activity of other growth factors, including hedgehogs, bone morphogenetic proteins, and fibroblast growth factors (69, 70). Presently, we cannot discard the possibility that at least in hepatocellular carcinomas that produce one or more of these growth factors, GPC3 is also stimulating cell proliferation by activating signaling pathways other than that regulated by canonical Wnts.

Recently, another group reported that antisense RNA-induced reduction of GPC3 expression stimulates cell proliferation in two hepatocellular carcinoma cell lines (HepG2 and Hep3B; ref. 71). In our laboratory, however, we have not been able to reproduce their results. In this regard, it is important to note that HepG2 cells have mutated β-catenin (72) and that the Wnt pathway is constitutively active in these cells. Because the constitutive activation occurs downstream of GPC3, it is expected that a reduction in GPC3 levels would not have an effect on Wnt signaling.

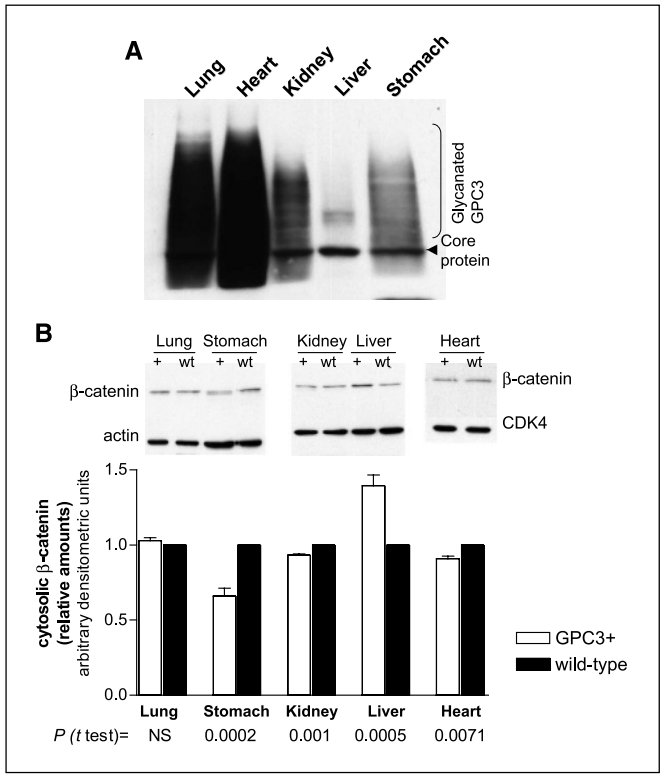

Figure 6. Differential effect of GPC3 on Wnt signaling in the liver. *A,* total lysates were prepared from the indicated organs obtained from GPC3-transgenic mice and GPC3 levels were assessed by Western Blot. *Right,* position of GPC3 core protein (*arrowhead*) and the glycanated GPC3 (*brace*). *B,* cytoplasmic levels of β-catenin from the indicated tissues of GPC3-transgenic (+) and wild-type littermate (*wt*) pairs of mice were analyzed by Western blot using actin or CDK4 as loading control. Each experiment was repeated with three pairs of mice from three different litters. The bands were scanned and quantified with a densitometer. *Columns,* average of β-catenin/loading control ratio; *bars,* ±SD. Statistical analysis was done by using unpaired *t* test (*P*s, *bottom*). A representative blot for each tissue (*top*).

Another important finding of this study is that at least in certain conditions, the heparan sulfate chains of GPC3 are not essential for the activation of the Wnt pathway. Until now, the predominant model was that the binding of glypicans to Wnts was mediated by their heparan sulfate chains (25). However, this model cannot explain previous reports showing that nonglycanated glypicans can have at least part of the biological activity of the wild-type counterparts (22, 23, 36). In fact, in this article, we show that in certain scenarios the core protein of GPC3 has as much biological activity as wild-type GPC3 (see Fig. 1*E* and Fig. 4*B* and *C*). In this regard, it is interesting to note that these scenarios include the *in vivo* tumorigenicity experiment and the *in vitro* experiments that used cocultured cells as source of Wnt. On the other hand, the GAG chains were required for optimal GPC3 activity in all the experiments that used soluble Wnts (see Fig. 1 and Fig. 4*A* and Fig. 5*A*). We speculate that the different roles of the GAG chains may be related to the way in which Wnts are presented to the hepatocellular carcinoma cells and/or to the relative Wnt concentration at the cell surface. Other factors that could have an effect on the role of the GAG chains include the type of Wnt and/or Frizzled involved. These factors could explain, for example, the fact that whereas the GPC3ΔGAG mutant stimulated cell proliferation and Wnt signaling in the PLC-PRF-5 cells, the same GPC3 mutant had no obvious activity in similar assays done in the HLF cells.

Our results indicate that GPC3 forms a complex with Wnts. This is consistent with the previous suggestion that glypicans act to facilitate the interaction between Wnts and their signaling receptors (Frizzleds and LRP5/6; ref. 25). If this is indeed the case, it is highly likely that these receptors may also be part of the glypican-Wnt complex. Additional studies will be required to confirm this hypothesis.

One of the major findings of this work is that the core protein of GPC3 can coimmunoprecipitate with Wnts. Because we also show that such core protein increases Wnt binding to the cell surface, we conclude that at least some Wnts can form a complex with GPC3 in the absence of heparan sulfate chains. Whether other proteins are required for the formation of the GPC3-Wnt complex remains to be investigated.

De Cat et al. recently reported that the GPC3ΔGAG mutant does not coimmunoprecipitate with Wnt5A in MCF-7 cells (23). At this point in time, it is not clear whether this discrepancy is due to the use of a different Wnt, or to the cellular context. It has to be noted, however, that in the same study, it was reported that the GPC3ΔGAG mutant has the same biological activity than the wild-type GPC3 (23).

The analysis of the GPC3-transgenic mice suggests that GPC3 displays a tissue-specific effect on Wnt signaling. In this regard, it is important to note that as discussed above, in some cellular systems, glypicans stimulate noncanonical Wnt signaling (21). We speculate that the tissue-specific effects of GPC3 may be determined by which type of Wnt signaling is predominantly active in a specific tissue. Thus, based on the assessment of the status of the Wnt pathway in the GPC3-transgenic mice, we

propose that in adult liver canonical Wnt signaling is dominant. On the other hand, because it is well established that activation of noncanonical Wnt signaling can reduce canonical activity, the inhibition of such signaling pathway in other organs of the GPC3-transgenic mice may reflect the predominance of the noncanonical Wnt pathway. In support of this hypothesis, we have recently shown that the noncanonical Wnt pathway is inhibited and canonical signaling activated in GPC3-null embryos (73). Alternatively, glypicans could act as direct negative regulators of canonical Wnt signaling in tissues where glypicans are predominantly released from the cell surface and where canonical Wnts are the limiting factor.

One of the implications of the work described here is that the manipulation of glypican expression can be used to regulate Wnt signaling. This discovery therefore has repercussions that go beyond hepatocellular carcinoma, because the Wnt pathway plays a critical role in various types of cancers.

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