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PRECLINICAL STUDY

Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation

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Abstract Glypican-3 (GPC3) is a proteoglycan involved in migration, proliferation and cell survival modulation in several tissues. There are many reports demonstrating a downregulation of GPC3 expression in some human tumors, including mesothelioma, ovarian and breast cancer. Previously, we determined that GPC3 reexpression in the murine mammary adenocarcinoma LM3 cells induced an impairment of their in vivo invasive and metastatic capacities together with a higher susceptibility to in vitro apoptosis. Currently, the signaling mechanism of GPC3 is not clear. First, it was speculated that GPC3 regulates the insulin-like growth factor (IGF) signaling system. This hypothesis, however, has been strongly challenged. Recently, several reports indicated that at least in some cell types GPC3 serves as a selective regulator of Wnt signaling. Here we provide new data demonstrating that GPC3 regulates Wnt pathway in the metastatic adenocarcinoma mammary LM3 cell line. We found that GPC3 is able to inhibit canonical Wnt signals involved in cell proliferation and survival, as well as it is able to activate non canonical pathway, which directs cell morphology and migration. This is the first report indicating that breast tumor cell malignant properties can be reverted, at least in part, by GPC3 modulation of Wnt signaling. Our results are consistent with the potential role of GPC3 as a metastasis suppressor.

Keywords β-Catenin · Cell–cell adhesion · Cell migration · Cell survival · Cytoskeleton · E-Cadherin · Glypican-3 · JNK · Wnt signaling

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Introduction

Glypicans are a family of heparan sulfate proteoglycans linked to the exocytoplasmic surface of the plasma membrane by a glycosyl-phosphatidylinositol anchor [1]. To date, six family members (GPC1 to GPC6) have been identified in mammals, and two in *Drosophila* [2–4]. In this latter model, glypicans have been shown to regulate Wnt pathway [5]. The involvement of glypicans in Wnt signaling has also been shown in Zebrafish and Xenopus [6, 7]. Currently two Wnt signaling pathways have been described in invertebrates and three in vertebrates [8]. In the canonical pathway, β-Catenin is first accumulated in the cytoplasm and, after translocation to the nucleus, it binds to LEF/TCF transcription factors, promoting expression of target genes [9] which include genes regulating cell proliferation and developmental processes as well as tumor progression. The non canonical planar cell polarity Wnt signaling (PCP), controls morphogenetic movements and planar cell polarity. This pathway triggers the activation of Rho small GTPases and c-jun N-terminal kinase (JNK), and regulates different cellular responses

that do not directly involve transcriptional events, such as tissue separation, lamellipodia formation, cytoskeleton rearrangements, and cell adhesion [8].

GPC3 is involved in migration, proliferation and modulation of cell survival in several tissues [10–13]. There are many reports demonstrating downregulation of GPC3 expression in several human tumors, such as mesothelioma, ovarian and breast cancer [14–16]. We have previously shown that GPC3 reexpression in the murine mammary adenocarcinoma LM3 cells leads to impairment of their in vivo invasive and metastatic capacities as well as higher susceptibility to in vitro induced apoptosis by stress conditions [13]. These results support the idea that GPC3 is likely to have a remarkable protective role towards breast cancer progression.

Currently, the mechanism by which GPC3 regulates cell proliferation and apoptosis is unclear. It has been proposed that GPC3 could be downregulating insulin-like growth factor (IGF) signaling [10, 17]. However, this hypothesis has been strongly challenged by other studies in mammalian systems, showing that GPC3 does not physically or genetically interact with the IGFs or their receptors [11, 18–20].

Recently, several reports indicated that, at least in some cell types, GPC3 serves as a selective regulator of Wnt signaling, modulating both the canonical and the non canonical pathways [21–24]. Here, we provide new data indicating that GPC3 regulates the Wnt pathway in the metastatic adenocarcinoma mammary LM3 cell line. We found that GPC3 is able to both inhibit the canonical Wnt signals involved in cell proliferation and survival, and to activate the non canonical pathway, by inhibiting migration and modulating cell morphology. Our results are consistent with a potential role for GPC3 as a metastasis suppressor.

Materials and methods

Tumor cell lines and cell culture procedures

LM3 cell line was established in our laboratory [25] from primary cultures of the spontaneous murine mammary adenocarcinoma M3 [26], however, in contrast to its parental tumor, LM3 cell line shows a highly metastatic in vivo behavior upon inoculation into syngeneic BALB/c mice. Cells were cultured at 37°C in a humidified 5% CO_2-air atmosphere, in minimum essential medium (MEM) (41500 Gibco BRL) with non essential aminoacids and 2 mM L-glutamine, and supplemented with 5% fetal calf serum (FCS) (Bioser) and 80 μg/ml gentamicin. Cells were periodically determined to be mycoplasma-free by the Hoechst's method.

We previously transfected the GPC3-negative LM3 cells with the pEF-BOS [27] vector containing a Hemaglutinin A (HA)-tagged OCI-5/GPC3 cDNA [28] or with the empty vector. LM3-GPC3 #1 and LM3-GPC3 #2 clones, expressing GPC3, and LM3-vector #1 and LM3-vector #2 control clones were chosen for the assays [13].

Immunoblotting

For preparation of total protein extracts, confluent monolayers were washed three times with ice cold PBS and then lysed with lysis Buffer (PBS-1% Triton X-100) containing a phosphatase inhibitor cocktail (Sigma) and protease inhibitors: 10 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin. Cell extracts were centrifuged at 15,000 rpm for 15 min at 4°C to remove insoluble materials. The resulting supernatants were collected and their protein concentration was determined by the Bradford method [29]. All samples were resusspended with an equal volume of 1× Laemmli loading buffer. Proteins were resolved by SDS-PAGE, transferred (25 V; 60 min) to PVDF membranes using the "Semidry-transfer method" (BioRad), and then analyzed by Western blotting using an anti-β-Catenin antibody (mouse monoclonal antibody clone 14, Transduction Laboratories), or an anti-phospho-JNK antibody (Cell Signaling) followed by incubation with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Sigma). Protein bands were detected using the ECL Reagent (Amersham Biosciences). Electrophoretic band images were obtained and analyzed by densitometry (OD) (Molecular AnalystTM GS-700, Bio-Rad). Total JNK levels were determined by stripping and reprobing the membranes with anti-JNK antibody (Cell Signaling).

For analysis of cytoplasmic β-Catenin levels, cytoplasmic extracts were obtained using a saponin buffer, as previously described [30]. Briefly, cells cultured in Petri dishes were lysed with 250 μl of saponin lysis buffer (25 mM Hepes, 75 mM potassium acetate, 0.1% saponin, phosphatase inhibitor cocktail and protease inhibitors). The extraction procedure was carried out twice; the extracts were pooled and then centrifuged before Western blotting with 5 μg/ml anti-β-Catenin antibody, followed by an anti-mouse horseradish peroxidase-conjugated antibody (Sigma). The levels of cytoplasmic β-Catenin were normalized with respect to the total β-Catenin levels.

Immunoprecipitation

For E-Cadherin and β-Catenin immunoprecipitation, LM3-GPC3 and LM3-vector cell lysates (500 μg protein) were precleared by incubating with protein G-agarose (Sigma) for 1 h at 4°C. Then, protein complexes were

immunoprecipitated from cleared lysates with either 2 μg of an anti-E-Cadherin antibody (Transduction Laboratories) or 2 μg of an anti-β-Catenin antibody, which were rocked overnight at 4°C. As a control of specificity, immunoprecipitation was also performed with 2 μg of a pre-immune mouse IgG (Sigma) or with a non specific antibody (anti-p27, C19, Santa Cruz Biotechnology). The immunocomplexes were then captured by adding protein G-agarose and rocked for an additional hour. Agarose beads were collected and washed three times with lysis buffer, then boiled for 10 min in sample buffer and subjected to Western blotting using anti-β-Catenin or anti-E-Cadherin antibodies, and detected as described above.

Immunofluorescence and confocal microscopy

LM3-GPC3 and LM3-vector cells were grown on glass coverslips. Cells were fixed in 4% formaldehyde/PBS at room temperature (RT), permeabilized with PBS-0.2% Triton X-100 for 15 min at RT and then blocked with PBS 5% BSA for 1 h at RT. E-Cadherin was detected by incubation with a rabbit polyclonal antibody dilution 1:200 for 2 h, followed by incubation with an anti-rabbit IgG-Alexa 568 secondary antibody for 1 h (Invitrogen Detection Technologies). β-Catenin was simultaneously detected with a mouse monoclonal antibody dilution 1:200 (Santa Cruz Biotechnology), followed by incubation with an anti-mouse-FITC secondary antibody (1:200, Zymed). Control experiments demonstrated no cross reactivity between the anti-mouse secondary antibody and rabbit primary antibody nor vice versa. Besides, no detectable staining was observed when the first antibody was missing. Images were obtained in a Carl Zeiss LSM 5 Pascal confocal microscope.

For the study of structural extracellular matrix components modulated by canonical Wnt signaling, the coverslips were incubated for 2 h at RT with an anti-Collagen I antibody (1:40, Dako). Next, cells were incubated for an hour with an anti-rabbit-FITC antibody (1:200, Zymed). Nuclei were counterstained with propidium iodide for 1 min prior to mounting with Vectashield (Vector).

In order to analyze the pathways involved in actin cytoskeleton organization, LM3-vector and LM3-GPC3 cells, either transiently transfected with RhoA N17, Rac N17 and Cdc42 N17 [31] or with the corresponding empty vectors, as well as also treated with 10 μM of a pharmacological JNK inhibitor (SP600125), were fixed, permeabilized, blocked and then incubated with 1:500 phalloidin-FITC (Sigma).

Homotypic adhesion

To evaluate whether GPC3 modulates cell–cell adhesion, 24-well plates were coated with a 1% agar (Gibco BRL) underlayer to prevent cell attachment. 3×10^4 LM3-GPC3 and LM3-vector cells, obtained from trypsinized log-phase growing monolayers, were treated (or not) with 7.5 μg of anti-E-Cadherin able to recognize the extracellular domain (Santa Cruz Biotechnology) for 30 min and then seeded in suspension in complete medium with 10% FCS. To control for specificity, cells were also incubated with a pre-immune mouse IgG. At 24 h, cultures were photographed and the spheroids formed were evaluated.

Wound-healing assay

To determine the effects of GPC3 reexpression on LM3 cell motility, a wound-healing assay was performed. Briefly, 5×10^5 cells were seeded on 35 mm plastic dishes in MEM supplemented with 10% FCS. Upon reaching cellular confluence was obtained, parallel wounds of about 400 μm width were applied to the monolayers. At time 0 and after 6 h of incubation in the presence or in the absence of the SP600125 pharmacological JNK inhibitor (10 μM), photographs of the same area were taken to determine wound coverage due to cellular motility. Images were obtained and evaluated by the image-Pro Plus 5.1 software.

Cell viability assay

To determine the role of JNK in GPC3 induced apoptosis, 10^5 cells were seeded in triplicates in 96-well plates, in 200 μl of complete medium supplemented with 5% FCS. At 24 h, cells were washed with PBS, and cultured in the absence of FCS for 24 additional hours. Monolayers were then treated with 5, 10 and 15 μM of the SP600125 pharmacological JNK inhibitor or with the vehicle (DMSO) only. At 48 h after treatment, viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product in viable cells (Cell Titer 96 TM, Promega Corp) as calculated by the 492/620 nm absorbance ratio.

Transient transfection

Gene reporter assays

To analyze the effects of GPC3 on JNK transcriptional activity, 7×10^4 cells/well were seeded in a 24-well plate and cotransfected with a Dual-Luciferase Reporter Assay (Promega) firefly-luciferase reporter vector driven by the AP-1 promoter (Stratagene) and with a renilla-luciferase vector using FuGene (Roche). At 36 h post-transfection, cells were lysed and luciferase activity was measured according to manufacturer's instructions.

Dominant negative mutants

To determine the role of RhoA, RacI and Cdc42 GTPases in GPC3-induced cytoskeleton reorganization, cells growing on 35 mm dishes were transfected with the dominant negative mutants: RacN17, RhoN17, Cdc42N17 [31] or the appropriate empty vector using FuGene (Roche). After 24 h, transfected cells were seeded on coverslips and grown up to subconfluence, and actin organization was evaluated as described above. As transfection control, protein extracts were obtained to evaluate GTPases' expression levels by Western blot.

PCR analysis

Total RNA extraction and cDNA synthesis

Total RNA was prepared from LM3-GPC3 and LM3-vector cells using the Trizol Reagent (Life Technologies Inc.) according to manufacturer's directions. RNA quantification and purity were assessed by measuring absorbance at 260 and 280 nm. Denaturing agarose gel electrophoresis was used to evaluate the quality of the samples.

Conventional reverse transcription reaction was used to yield single-strand cDNA. The first-strand cDNA was synthesized from 1 μg total RNA, previously treated with 1 unit of DNase I (FPLC-pure, Amersham Biosciences), using: random and oligo(dT) primers, RNase inhibitor and SuperScript II reverse transcriptase, all according to manufacturer's recommendations (Invitrogen Life Technologies). The resulting cDNA was then treated with 1 unit of RNase H (Amersham Biosciences) and diluted 1:4 with TE buffer. Controls for the absence of self-priming were obtained by performing reverse transcription in the absence of primers, and controls for the absence of genomic DNA contamination were obtained by incubation with primers in the absence of reverse transcriptase enzyme.

Reverse transcription polymerase chain reaction (RT-PCR)

cDNA obtained as described above was incubated with different pairs of Wnt gene-specific primers [24]. PCRs were completed in the presence of 150 nM MgCl and 200 nM dNTPs using the following conditions: denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and elongation at 72°C for 10 min. Embryo RNA was used as a positive control. The PCR amplified products were visualized after ethydium bromide staining on an agarose gel.

Quantitative real time PCR (qPCR)

The expression levels of selected genes were determined by qPCR analysis. Primers were designed to amplify 100–150 bp length amplicons, with a melting temperature of 60°C, and then synthesized by Invitrogen. Analysis of DNA melting curves demonstrated a single peak for the whole set of primers. Primer sequences were as follows (5′ to 3′):

CD44 F: CCAACACCTCCCACTATGAC
R: TATACTCGCCCTTCTTGCTG

E-Cadherin F: AAGTGACCGATGATGATGCC
R: CTTCATTCACGTCTACCACGT

β-Catenin F: GGGATGTTCACAACCGAATTGT
R: GCTACTCTTTGGATGTTTTCAATGG

For each transcript, cDNAs were analyzed in replicates by qPCR. All qPCR reactions were carried out in a volume of 25 μl containing: 2 μl of cDNA, 12.5 μl 2X SYBR Green I Master Mix (Applied Biosystems), and forward and reverse primers to a final concentration of 800 nM. Reactions were run on an ABI Prism 5700 sequence detector (Applied Biosystems). The cycle conditions comprised a 10 min period of polymerase activation at 95°C, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. We tested five hosekeeping genes (tubulin, actin, HPRT, 36B4, GAPDH). Since GAPDH gene presented less expression variability between clones, for quantitative results the levels of each transcript were normalized to the level of internal housekeeping control GAPDH gene (F: ACCCACTCCTCCACCTTTGA and R: CTGTTGCTGTAGCCAAATTCGT) and represented as fold change using the ΔCt method [32].

Statistical analysis

All experiments were performed at least in triplicate using two LM3-GPC3 (LM3-GPC3 #1 and LM3-GPC3 #2) clones and two LM3-vector (LM3-vector #1 and LM3-vector #2) clones. The significance of differences between groups was calculated by applying ANOVA/Bonferroni's tests as indicated. A value of $P < 0.05$ was considered to be significant.

Results

GPC3 reexpression leads to inhibition of the canonical Wnt signaling

Total and cytoplasmic β-Catenin

Since β-Catenin is a key molecule in the canonical Wnt pathway, we first studied its expression and localization. We determined that β-Catenin expression is significantly

higher, both at the mRNA and protein levels, in LM3-GPC3 than in LM3-vector clones (Fig. 1a, b). However, when the cytoplasmic pool of β-Catenin was analyzed by Western blot, we found a cytoplasmic/total β-Catenin ratio around 8 times lower in LM3-GPC3 cells (Fig. 1a). This result suggests that GPC3 reexpression is inducing an inhibition of canonical Wnt signaling.

β-Catenin/E-Cadherin association

β-Catenin is able to associate with E-Cadherin at the membrane level forming cell–cell adhesion complexes. Using qPCR, we found that GPC3 expressing cells showed higher E-Cadherin mRNA levels (Fig. 1c). Therefore, to establish whether this adhesion molecule played a role in the redistribution of β-Catenin, we set out to evaluate the E-Cadherin-linked β-Catenin pool by immunoprecipitation. As shown in Fig. 2a, when protein extracts were immunoprecipitated with an anti-E-Cadherin antibody, higher levels of co-immunoprecipitated β-Catenin were found in LM3-GPC3 cells. The same results were obtained when the immunoprecipitation-immunoblotting procedure was reversed (data not shown). Neither E-Cadherin nor β-Catenin were detected in immuno-complexes from pre-immune IgG immunoprecipitations, demonstrating the specificity of the interaction.

Further support for the existence of a higher number of E-Cadherin/β-Catenin complexes in LM3-GPC3 cells was provided by an immunocolocalization assay using confocal microscopy. As it can be seen in Fig. 2b, β-Catenin was diffusely localized in LM3-vector cells body, while E-Cadherin was mainly localized in the membrane. In contrast, GPC3 reexpression induced the redistribution of β-Catenin towards the plasma membrane, colocalizing with E-Cadherin. In addition, while LM3-vector cells presented an important nuclear staining for β-Catenin, the nuclei of

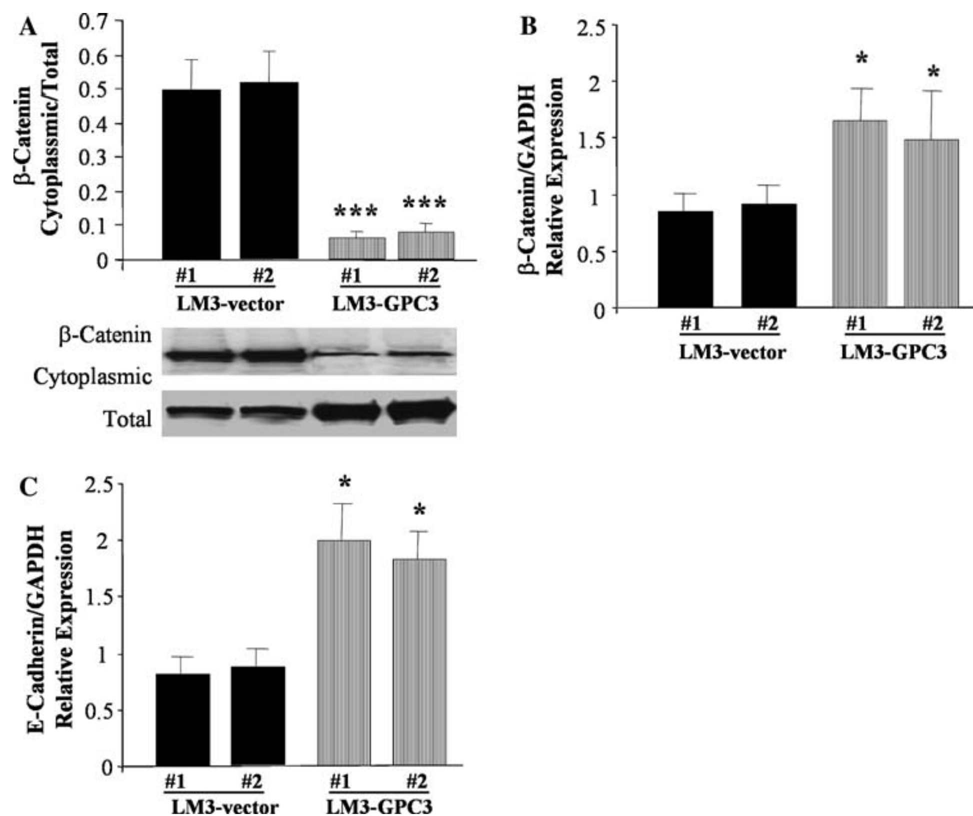

Fig. 1 Effects of GPC3 reexpression on β-Catenin and E-Cadherin levels. (**a**) Cytoplasmic and total protein extracts were obtained from LM3-GPC3 and LM3-vector cells. Extracts were analyzed by Western blot using an anti β-Catenin antibody. Loading was standardized by actin levels of each fraction. Bars represent the mean ± SD of the cytoplasmic/total levels ratio of β-Catenin. Values were obtained from duplicate measures, in three independent experiments. A representative western blot is shown. The difference in cytoplasmic/total β-Catenin ratio was statistically significant to a $P < 0.005$ by ANOVA/Bonferroni's tests (***). (**b** and **c**) Total RNA from control and GPC3 expressing cells were retrotranscripted and analyzed by three replicate measurements in quantitative real-time PCR (qPCR) reactions. The level of each transcript was normalized to the level of internal housekeeping control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Bars represent the mean ± SD of the β-Catenin/GAPDH (**b**) and E-Cadherin/GAPDH (**c**) ratio. The difference was statistically significant to a $P < 0.05$ by ANOVA/Bonferroni's tests (*)

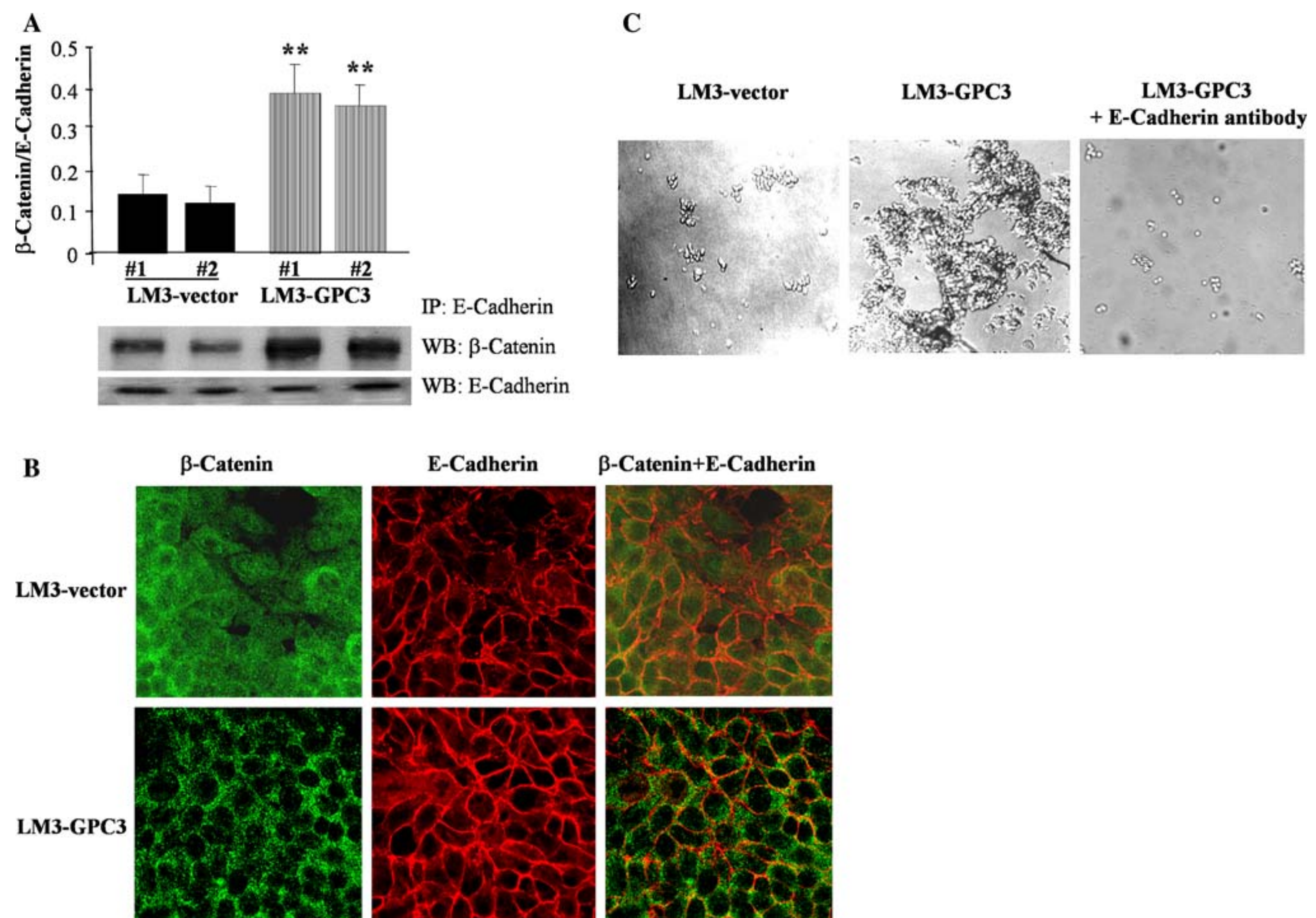

Fig. 2 Effects of GPC3 reexpression on β-Catenin/E-Cadherin association. (**a**) E-Cadherin was immunoprecipitated from LM3-GPC3 and LM3-vector cells. Immunoprecipitated proteins were then analyzed by Western blot using an anti-β-Catenin antibody. Bars represent the mean ± SD of β-Catenin/E-Cadherin ratio. Values were obtained from duplicate measures, in three independent experiments. A representative Western blot is shown. The difference between clones was statistically significant to a $P < 0.01$ by ANOVA/Bonferroni's tests (**). (**b**) LM3-GPC3 and LM3-vector cells were seeded on coverslips and then fixed. β-Catenin (green) and E-Cadherin (red) were detected as described in 'Materials and methods' using confocal microscopy. Same cells are shown in each row and images on the right panel are formed by superimposition of the other two panels in the same row. GPC3 induced colocalization of β-Catenin and E-Cadherin is denoted by yellow color. Images shown here are representatives of three experiments. 400× magnifications. (**c**) LM3-GPC3 and LM3-vector cells were treated or not with an anti-E-Cadherin extracellular domain antibody and then seeded in suspension. At 24 h, cultures were photographed and the spheroids formed were evaluated

GPC3 expressing cells were negative for this molecule. These results confirm the idea that GPC3 would be inducing an inhibition of canonical Wnt pathway.

Cell–cell adhesion

We analyzed whether GPC3 modulates homotypic cell–cell adhesion and the possible involvement of GPC3-induced E-Cadherin upregulation on this behavior. As shown in Fig. 2c, LM3-GPC3 cells formed large cohesive clusters of more than 50 cells when the attachment to a substrate was impaired, while LM3-vector cells remained single or only formed small clusters. Interestingly, when cells were treated with a neutralizing anti-E-Cadherin antibody, LM3-GPC3 clones notably reduced their cell–cell adhesion ability (Fig. 2c), and thus corroborating the role of E-Cadherin in the enhanced cell–cell adhesion ability of LM3-GPC3 cells.

Expression of canonical Wnt target genes

To confirm that GPC3 is inhibiting canonical Wnt pathway, we analyzed the expression of two of its target genes: type I collagen and CD44. Canonical Wnt signaling activation inhibits the expression of different collagens [33] and stimulates the transcription of the receptor for the hyaluronic acid CD44. We observed, by means of immunofluorescence, that GPC3 reexpression induced a remarkable increase of type I collagen fibers (Fig. 3a). On the other side, CD44 mRNA levels were significantly reduced in GPC3 expressing cells as evaluated by qPCR (Fig. 3b). These results give

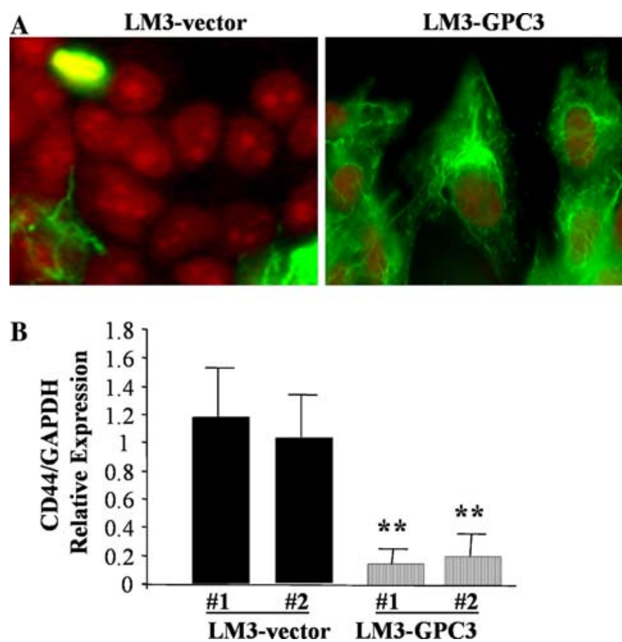

Fig. 3 Effects of GPC3 reexpression on canonical Wnt target genes. (**a**) Cells seeded on coverslips were fixed and incubated with an anti-Collagen I antibody, followed by a—FITC secondary antibody. Nuclei were counterstained with propidium iodide. Control experiments with secondary antibodies only did not present any detectable staining. 1000×. (**b**) Total RNA from control and GPC3 expressing cells were retrotranscripted and analyzed for CD44 mRNA expression by three replicate measurements in qPCR reactions. The level of each transcript was normalized to the level of internal housekeeping control GAPDH gene. Bars represent the mean ± SD of the CD44/GAPDH ratio. The difference was statistically significant to a $P < 0.01$ by ANOVA/Bonferroni's tests (**)

further support to our idea that GPC3 is inducing a functional inhibition of canonical Wnt pathway.

GPC3 induces activation of the non canonical Wnt signaling

JNK activity

We decided to investigate whether GPC3 expressing cells also exhibited alterations in the non canonical Wnt pathway, studying JNK activation.

LM3-GPC3 cells exhibited a 4.5-fold increase in JNK phosphorylation, while no changes were found in the total amount of JNK (Fig. 4a). These results suggest that the non canonical Wnt signaling is stimulated by GPC3 reexpression in LM3 mammary tumor cells. We then investigated whether JNK was involved in the higher susceptibility to apoptosis showing by the LM3-GPC3 cells [13]. It was found that the inhibition of JNK by SP600125 did not modify LM3-GPC3 cells death in serum-deprived conditions (Fig. 4b). These results suggest that even though JNK is more active, it is not implicated in the GPC3-induced apoptosis.

Besides, it is known that JNK participates in modulation of the AP-1 transcription factor activity [34]. So, we performed a gene reporter assay using a luciferase reporter vector driven by the AP-1 promoter. We observed that the increased JNK phosphorylation detected in LM3-GPC3 cells did not lead to higher AP-1 activity (Fig. 4c). Therefore, JNK activation induced by GPC3 may have other effects that do not directly involve transcriptional events.

Cytoskeleton organization and cell migration

The non canonical PCP Wnt pathway is implicated in morphogenetic processes, which are involved in cytoskeleton organization and in epithelial cell polarity [35]. Therefore, we studied the actin distribution using phalloidin-FITC staining. As shown in Fig. 5, while the LM3-vector cells displayed a high number of stress fibers across the cell body as expected for tumor cells that underwent epithelial to mesenchymal transition (EMT) [36], GPC3 reexpression induced cytoskeletal reorganization, with a reduction of these fibers, and most of the F-actin localizing to a cortical position. Interestingly, when JNK activation was blocked, the LM3-GPC3 cells recovered their stress fibers. These results support the hypothesis that GPC3 induces actin cytoskeleton reorganization through non canonical Wnt signaling.

We then assayed the effects of some members of the Rho family of small GTPases (RhoA, Racl, and Cdc42) [37], which are known to participate in cytoskeleton organization induced by the non canonical Wnt pathway [6, 38–41]. LM3-GPC3 and LM3-vector cells were transfected with expression vectors encoding for dominant negative mutants of these GTPases. Although these mutant GTPases induced the expected changes in the actin cytoskeleton, no differences were found between LM3-GPC3 and LM3-vector cell clones (data not shown). It is possible that the GPC3-induced modulation of the PCP pathway regulates cytoskeleton changes through other JNK activating GTPases [42].

Since cytoskeleton organization is an important determinant of the cell migratory capacity, we decided to evaluate the role of the non canonical Wnt signaling on GPC3 modulated cell migration. By means of a wound-healing assay, and in agreement with previous results [13], we determined that LM3-GPC3 cells were significantly less migrating than the control ones. Interestingly, the inhibition of JNK was able to revert this decreased migration displayed by the LM3-GPC3 cells (Fig. 6).

LM3 cells express canonical Wnt 2B and Wnt 7A and non canonical Wnt 5A, Wnt 4 and Wnt 11

The results here presented suggest the existence of an autocrine-Wnt modulation. To assess which Wnt proteins

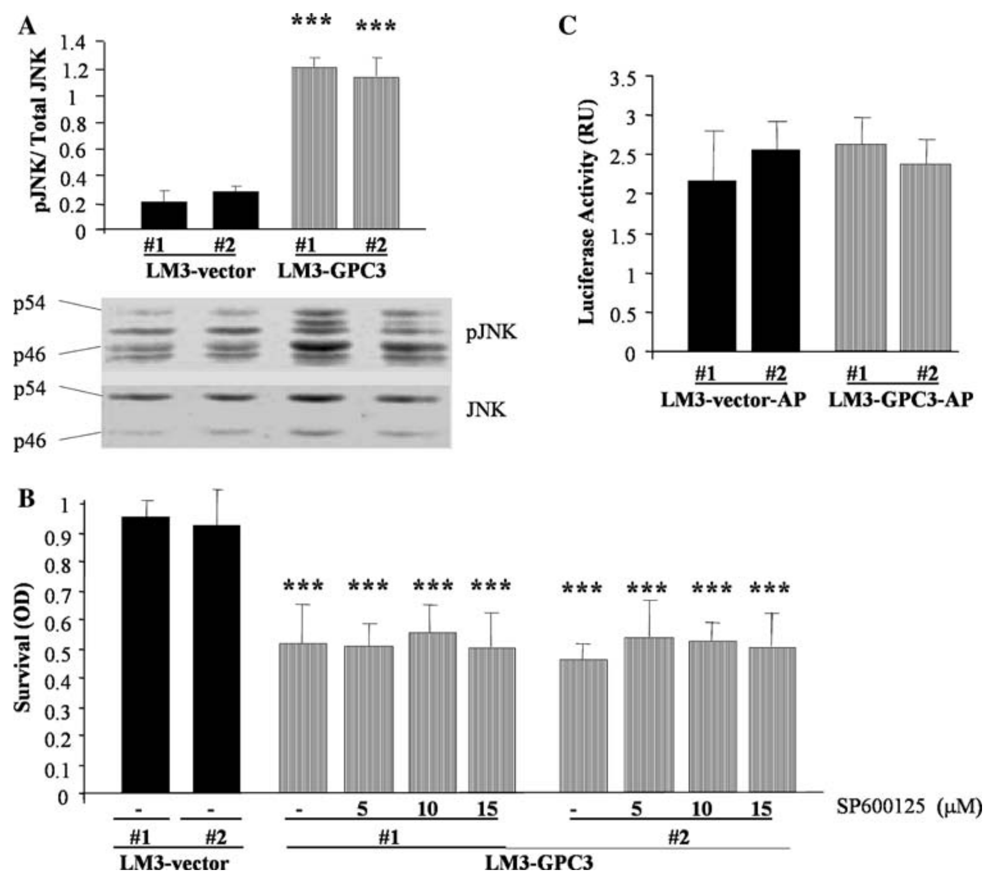

Fig. 4 Effects of GPC3 reexpression on non canonical Wnt signaling. (**a**) Protein extracts of GPC3-transfectans and control clones were subjected to Western blot for total and phospho-JNK. Bars represent the mean ± SD of phospho-JNK/JNK ratio levels. Values were obtained from duplicate measures, in three independent experiments. A representative Western blot is shown. The difference between clones was statistically significant to a $P < 0.005$ by ANOVA/ Bonferroni's tests (***). (**b**) Subconfluent monolayers were starved over night, and then treated with the pharmacological JNK inhibitor SP600125, or with the vehicle (DMSO). Viability was assessed by MTS 48 h after treatment, determining the absorbance 492/620 nm (OD). Bars represent the mean ± SD of samples OD. Values were obtained from duplicate measures of three independent sets of clones. The difference between LM3-vector and LM3-GPC3 (treated or not) cells was statistically significant to a $P < 0.005$ by ANOVA/ Bonferroni's tests (***). (**c**) LM3-GPC3 and LM3-vector cells were cotransfected with a firefly luciferase reporter vector driven by the AP-1 promoter and with a renilla-luciferase vector. Luciferase activity was measured and presented in this histogram. Bars represent the mean ± SD of triplicates. Values were obtained from triplicate measures, in three independent experiments

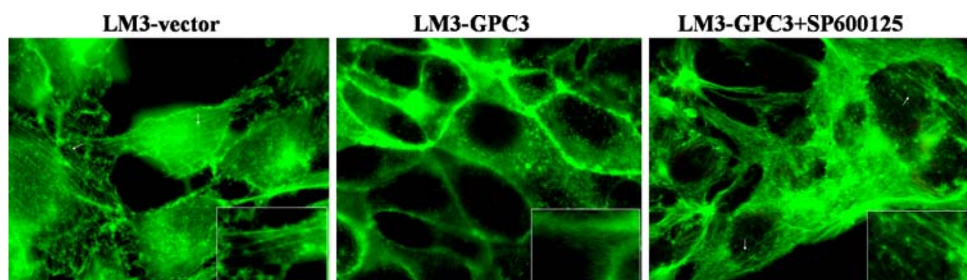

Fig. 5 Effects of GPC3 reexpression on actin cytoskeleton organization. LM3-vector and LM3-GPC3 cells treated or not with 10 μM of SP600125, were fixed, permeabilized, blocked and then incubated with phalloidin-FITC. Fluorographic pictures of filamentous actin (F-actin) are representative. 1000×

are endogenously expressed, and whether the differences in Wnt signaling activity could be due to changes in the expression pattern of Wnt factors, we performed RT-PCRs. We found that all clones express the canonical Wnt 2B and Wnt 7A and the non canonical Wnt 5A, Wnt 4 and Wnt 11 (Fig. 7), without any difference between the LM3-GPC3

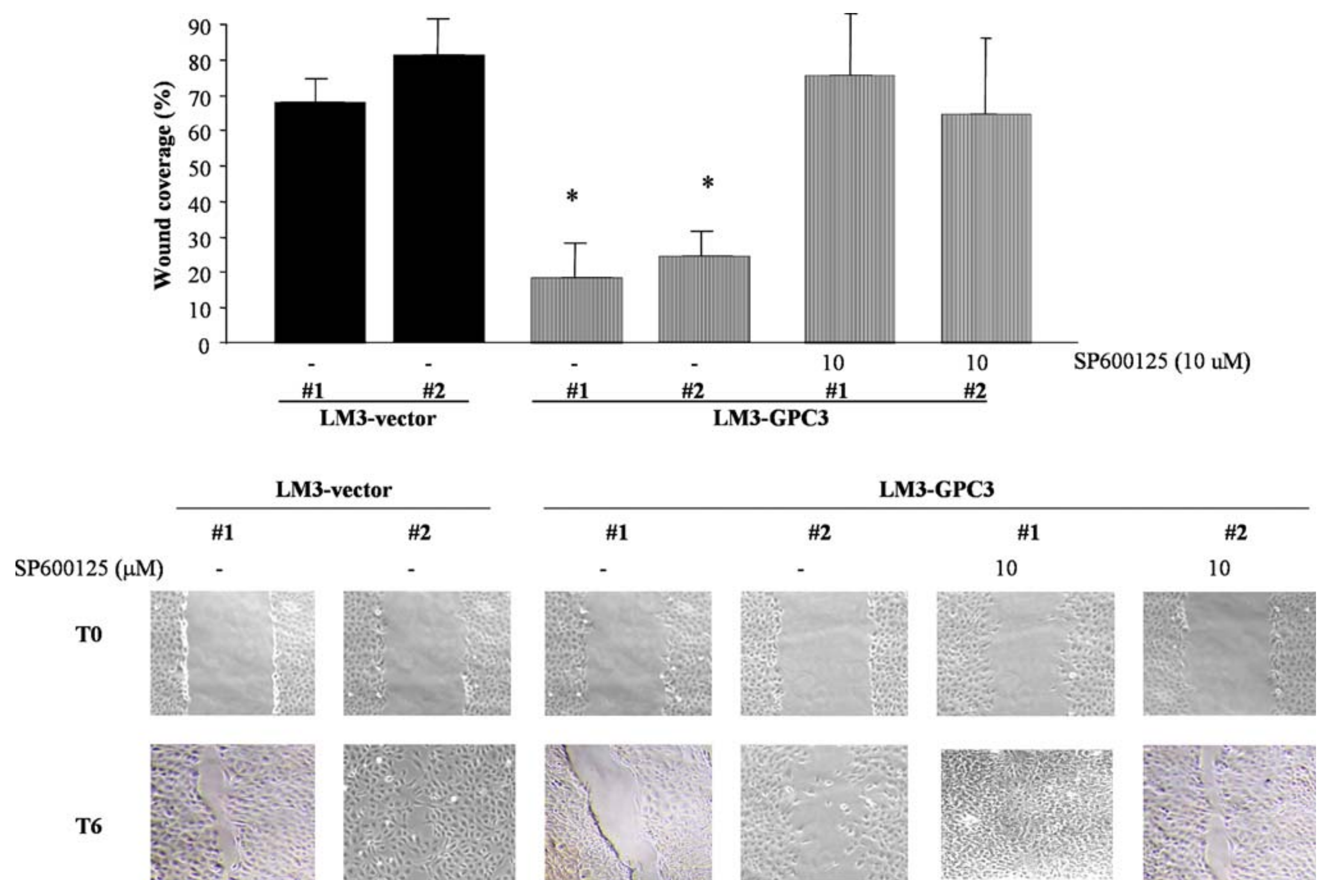

Fig. 6 Effects of GPC3 reexpression on cell migration. A wound-healing assay was performed. Once cellular confluence was obtained, parallel wounds of about 400 μm wide were done on monolayers. At time 0 (t0) and after 6 h (t6) of incubation in presence or absence of SP600125 (10 μM), photographs of the same area were taken to determine wound coverage due to cellular motility. Images were obtained and evaluated by the image-Pro Plus 5.1 software. Bars represent the mean ± SD of wound coverage (%). Values were obtained from duplicate measures of three independent experiments. Representative photographs are shown. The difference between clones was statistically significant to a $P < 0.05$ by ANOVA/Bonferroni's tests (*)

and LM3-vector cells. These findings suggest that GPC3 could be modulating the canonical and the non canonical Wnt pathways by regulating the autocrine activity of the different Wnt factors.

Discussion

It has been shown that GPC3 is able to modulate tissue growth and development; therefore, it is not surprising that alterations in its function are associated to cell transformation and tumor progression. Interestingly, deregulation of GPC3 expression can either inhibit or activate tumor growth, depending on the cellular context. In particular, the loss of GPC3 expression seems to be a key event in mammary gland malignant transformation [13, 16]. We previously determined that GPC3 acts as a metastasis suppressor in LM3 adenocarcinoma mammary cells, inducing changes in the cellular behavior toward a less invasive and metastatic phenotype [13]. Given that metastasis dissemination is the event that overshadows the oncology patient prognostic, we believe that the study of the molecular mechanisms involved in the effects of GPC3 has important clinical implications for breast cancer therapy.

Experiments in mammals have demonstrated that glypicans are required for Wnt activity. Since the Wnt signaling pathway can regulate, among other functions, adhesion, growth and cellular differentiation, it is essential to study its role in tumor progression [9].

It has been reported that glypicans can regulate canonical as well as non canonical Wnt signaling [20, 22, 23, 43]. Here we provide new evidence indicating that GPC3 regulates the Wnt pathway, by inhibiting canonical Wnt signals and activating non canonical ones in mammary tumor LM3 cells.

Canonical Wnt activation has been linked to stimulation of cell proliferation and inhibition of apoptosis [22, 23]. In agreement with the results obtained by Song et al. [24], we found a reduction of the β-Catenin cytoplasmic pool in

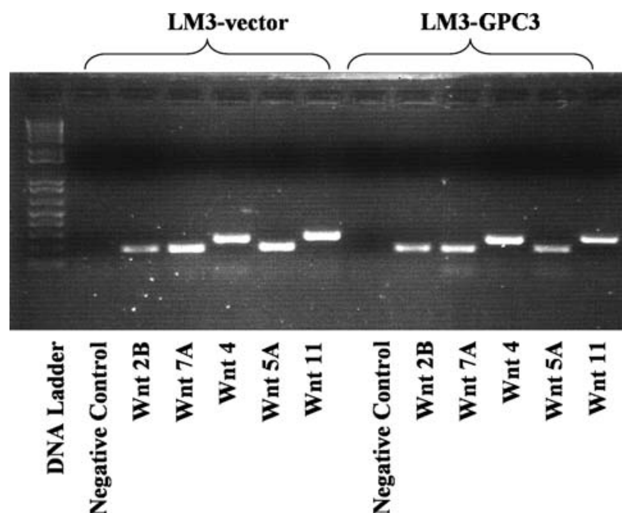

Fig. 7 Expression of Wnt factors. RNA from LM3-GPC3 and LM3-vector cells was subjected to RT-PCR by using different pairs of Wnt gene-specific primers. PCR products were resolved unto agarosa gel and stained with ethidium bromide. Embryo RNA was used as a positive control. As negative control, the RNA was replaced with water. In the graph a representative gel is shown

LM3-GPC3 cells, indicating that GPC3 reexpression induced inhibition of the canonical Wnt pathway. The reduction of nuclear β-Catenin detected by immunofluorescence is in agreement with this idea. Besides, this canonical pathway inhibition could be associated with the higher apoptosis rate detected in LM3-GPC3 cells [13].

These results could seem to be contradictory with those presented by Capurro et al. [22], who reported a GPC3 overexpression in hepatocarcinoma cells. In that experimental model, GPC3 induces a stabilization of cytoplasmic β-Catenin and activates the canonical Wnt pathway. Opposite to an apparent contradiction, their results reinforce the role of GPC3 in the modulation of Wnt pathways. The differences in the activation/inhibition of the Wnt sub-pathways are tissue-specific, and are based on the differential functions of GPC3 in each tissue.

Catenins also associate with Cadherins at the membrane level, forming adhesion complexes. Most malignant carcinomas develop dysfunctional cell adhesion, which results in the loss of epithelial morphology and increased cell motility and invasiveness [44]. We observed that GPC3 expressing cells presented a higher grade of β-Catenin/E-Cadherin association. In addition, we have shown that LM3-GPC3 cells present more E-Cadherin, both at the mRNA and protein levels. Since this molecule is down-regulated by the transcription complex β-Catenin/LEF/TCF [45], we suggest that inhibition of the canonical Wnt pathway induces increased E-Cadherin transcription in LM3-GPC3 cells. In turn, enhanced levels of E-Cadherin could sequester more β-Catenin molecules to the membrane, inhibiting their signaling function and generating a negative feedback loop.

Since β-Catenin is part of these cell–cell adhesion complexes, inhibition of the canonical Wnt signaling activity would implicate in increased intercellular adhesion [46]. LM3-GPC3 cells presented higher homotypic adhesion than control cells and we determined that this behavior is probably due to an enhanced number of E-Cadherin/β-Catenin complexes. These results are associated to the less aggressive in vivo phenotype displayed by the GPC3 expressing cells [13]. Moreover, the presence of a higher number of β-Catenin/E-Cadherin complexes at the plasma membrane level in LM3-GPC3 cells could explain the less motile phenotype shown by these cell clones.

The inhibitory effect of GPC3 on canonical Wnt pathway was also reflected at transcriptional level, since the expression of target genes, such as CD44 and type I Collagen, was remarkably modulated in LM3-GPC3 cells. The above mentioned modifications in adhesion molecules and matrix components could seriously compromise the metastatic capacity of LM3 cells [47].

Non canonical PCP Wnt signaling is involved in important events during vertebrates embryogenesis regulating cytoskeleton organization and cellular motility [39]. It has been described that GPC3 induces changes in this non canonical Wnt pathway, by either stimulating or inhibiting its activity, depending on the cellular context [24]. In this report we demonstrated that GPC3 reexpression induced a significant increase of phospho-JNK levels, suggesting an activation of the non canonical Wnt signaling. De Cat et al. [23] demonstrated that JNK activation is critical for GPC3-induced apoptosis in MCF-7 cells. In contrast, in our model the GPC3-induced JNK activation was not directly linked to cell death or survival. It is known that upon JNK activation, a fraction of these molecules enters the nucleus, where they phosphorylate several transcription factors, such as AP-1, resulting in modulation of transcriptional activity [48, 49]. Here, we did not find any difference in the activity of the transcription factor AP-1 between LM3-GPC3 and LM3-vector cell clones. These results are in agreement with the idea that JNK has a cytoplasmic role, in addition to its established nuclear functions [50].

Since the cytoskeleton reorganization induced by the non canonical Wnt pathway is driven by the Rho family of small GTPases, as well as by JNK itself [39], we speculated that the differential JNK activation found would be mediating other cellular events such as actin restructuring [51]. To this effect, we demonstrated that GPC3 reexpression induced actin redistribution and localization mainly to the cell cortical region and caused a significant reduction in the number of stress fibers. These modifications were dependent on JNK activation. The role of this kinase in the regulation of stress fibers has previously been reported. In fact, JNK

inhibition seems to cause the appearance of stress fibers around cell edges in some cell types, which indicates that JNK activation might suppress stress fibers formation [52]. Therefore, we propose that changes in the F-actin organization might also be responsible, together with enhanced cell–cell adhesion, for the reduction in cell migratory capacity detected in the GPC3 expressing clones. Supporting this hypothesis, we observed a significant increase in cell motility when LM3-GPC3 cells were treated with a JNK pharmacological inhibitor. Therefore, in addition to the conventional roles of JNK in inflammation, differentiation [53] and apoptosis [54], currently evidence is also accumulating to implicate JNK in cell migration. Xia and Karin recently reviewed the physiological role of JNK in regulating cell migration during epithelial morphogenesis in *Drosophila* and mice [55]. Other authors have reported that JNK activation correlates with increased migration in several cell types [51]. However, our results indicate that JNK is inhibiting migration in LM3 cells. These contradictory findings might reflect cell type differences in the regulation of migration by JNK, requiring further investigation. In summary, GPC3 would be inhibiting LM3 cells migratory capacity through the activation of the non canonical Wnt pathway, which causes JNK phosphorylation-mediated actin cytoskeleton reorganization.

It is well known that during tumor development, cells undergo an epithelial to mesenchymal transition (EMT), in which the epithelial phenotype, polarity and actin organization are lost and a mesenchymal phenotype is acquired [56]. Our results allow us to propose that GPC3 is able to induce the opposite effect, a mesenchymal to epithelial transition (MET) [57], normalizing LM3 cells to a more differentiated phenotype.

Since we found that GPC3 reexpression modulates Wnt pathways in LM3 mammary tumor cells, we suggest that this proteoglycan is modulating an already existent autocrine type of Wnt signaling. The fact that LM3 mammary adenocarcinoma cells express activators of both canonical (Wnt 2B and Wnt 7A) and non canonical signaling (Wnt 5A, Wnt 4 and Wnt 11), confirmed the existence of an autocrine system. In addition, GPC3 appears to have no effect on the Wnts pattern of expression in our system, suggesting that differential expression of Wnt factors is not the mechanism involved in modulation of GPC3 signaling. Other authors have detected expression of *wnt* genes in human breast tumors. Thus, Bafico et al. have reported that autocrine-Wnt activity is increased in several cancers, but the mechanism underlying this enhancement has not been established so far [58].

Our results provide new evidence supporting the idea that GPC3 modulates Wnt pathways. This is the first report demonstrating that breast tumor cell malignant properties can be reverted, at least in part, by GPC3 modulation of Wnt signaling. GPC3 is probably regulating cellular morphology, adhesion and extracellular matrix formation through Wnt signaling in this murine mammary cancer model. Furthermore, the alterations induced by GPC3 expression have important consequences in LM3 cells behavior, since they develop a less aggressive phenotype both in vivo and in vitro, probably due to the reversion of different altered parameters. We believe that understanding of these mechanisms will greatly contribute to understanding how GPC3 inhibits metastasis, being of potential application to breast cancer therapy.

Finally, another implication of this work is that manipulation of GPC3 expression can be used to regulate Wnt signaling. This discovery has a profound impact that goes beyond breast cancer, because Wnt pathways play a critical role in several tumor types.

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