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Authors: C. Buchanan · I. Stigliano · H.M. Garay-Malpartida · L. Gomes Rodrigues · L. Puricelli · M.C. Sogayar · E. Bal de Kier Joffé · M.G. Peters

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Abstract

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Keywords (separated by '-')	Glypican-3 - Apoptosis - PI3K/Akt pathway - p38MAPK pathway - Breast cancer
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PRECLINICAL STUDY

Glypican-3 reexpression regulates apoptosis in murine adenocarcinoma mammary cells modulating PI3K/Akt and p38MAPK signaling pathways

C. Buchanan · I. Stigliano · H. M. Garay-Malpartida · L. Gomes Rodrigues · L. Puricelli · M. C. Sogayar · E. Bal de Kier Joffé · M. G. Peters

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Abstract Glypican-3 (GPC3) is a proteoglycan involved in proliferation and cell survival. Several reports demonstrated that GPC3 is downregulated in some tumors, such as breast cancer. Previously, we determined that GPC3 reexpression in the murine mammary adenocarcinoma LM3 cells induced an impairment of their invasive and metastatic capacities, associated with a decrease of their motility and an increase of their cell death. We demonstrated that GPC3 inhibits canonical Wnt signaling, as well as it activates non canonical pathway. Now, we identified signaling pathways responsible for the pro-apoptotic role of GPC3 in LM3 cells. We found for the first time that GPC3 inhibits the PI3K/Akt anti-apoptotic pathway while it stimulates the p38MAPK stress-activated one. We report a concomitant modulation of CDK inhibitors as well as of pro- and anti-apoptotic molecules. Our results provide new clues regarding the mechanism involved in the modulation induced by GPC3 of mammary tumor cell growth and survival.

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Keywords Glypican-3 · Apoptosis · PI3K/Akt pathway · p38MAPK pathway · Breast cancer

Introduction

Glypicans are heparan sulfate proteoglycans that are bound to the cell surface through a glycosylphosphatidylinositol (GPI) anchor [1]. To date six family members (GPC1–GPC6) have been identified in mammals, and two of them in Drosophila [2, 3]. Although the specific functions of glypicans are still not completely known, it has been suggested that GPC3 inhibits cell proliferation and survival [3–5].

The Simpson-Golabi-Behmel Syndrome (SGBS) is an X-linked disorder characterized by pre- and post-natal overgrowth, visceral and skeletal anomalies, and an increased risk for the development of embryonic tumors [6]. Pilia et al. [7] reported that individuals with SGBS display mutations in the *OCI-5/GPC3* gene. In addition, GPC3 knockout mice exhibit several features of SGBS, including somatic overgrowth, renal dysplasia, accessory spleens, polydactyly, and placentomegaly [4, 5]. These findings, together with cell line-specific promotion of apoptosis by GPC3 [8], suggest that GPC3 plays a negative role in cell proliferation and also an apoptosis-inducing function in specific tissues.

GPC3 expression is frequently silenced by promoter methylation in mesotheliomas and ovarian and breast cancer [9–11]. In addition, GPC3 ectopic expression in some cell lines derived from these cancers inhibited cell growth, suggesting a role of GPC3 as a tumor suppressor [11]. Interestingly, we have shown that GPC3 reexpression in the murine mammary adenocarcinoma LM3 cell line leads to an impairment of its in vivo invasive and

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metastatic capacities associated with a significant decrease of its in vitro migration ability and an increase of its susceptibility to stress induced cell death [12]. These results support the idea that GPC3 has a remarkable protective role towards breast cancer progression.

Currently, the mechanism by which GPC3 regulates cell proliferation and survival is not clear. It has been speculated that GPC3 may regulate insulin-like growth factor (IGF) signaling, serving as a negative regulator of this pathway [13]. This hypothesis, however, has been strongly challenged by other studies in mammalian systems, showing that GPC3 does not interact physically or genetically with the IGFs or their receptors [4, 14, 15]. On the other side, several reports indicated that, at least in some cell types, GPC3 serves as a selective regulator of Wnt signaling [16, 17]. In this sense, we have recently provided 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 regulates cell morphology and migration [18].

Here we identify signaling regulatory pathways responsible for the pro-apoptotic role of GPC3 in LM3 cells. We found for the first time that GPC3 inhibits the PI3K/Akt anti-apoptotic pathway while it stimulates the p38MAPK stress-activated one. In addition, we report a concomitant modulation of cyclin-dependent kinase inhibitors as well as of pro- and anti-apoptotic molecules induced by GPC3 reexpression. Our results provide new and important clues regarding the mechanism involved in the modulation induced by GPC3 of mammary tumor cell growth and survival.

Materials and methods

Tumor cell lines and cell culture procedures

LM3 cell line was established in our laboratory [19] from primary cultures of the spontaneous murine mammary adenocarcinoma M3 [20]. 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 [21] vector containing a Hemagglutinin A (HA)-tagged OCI-5/GPC3 cDNA or with the empty vector [22]. LM3-GPC3 #1 and LM3-GPC3 #2 clones expressing GPC3 and LM3-vector #1 and LM3-vector #2 control clones were chosen for the following assays [12].

Flow cytometry

Subconfluent monolayers growing in 35 mm plates were depleted of FCS for 48 or 96 h and treated or not with the pharmacological p38MAPK inhibitor SB202190 (15 μM). Trypsinized cells were incubated with Ligation Buffer (10 mM Hepes (pH = 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing Annexin-V+ FITC (1:5000) and with propidium iodide (PI) (100 μg/ml).

Twenty minutes post-incubation at room temperature (RT), and protected from light, the samples were examined in a FACS calibur cytometer (Becton Dickinson). The results were analyzed using the Cell Quest Program.

Cell viability assay

To determine the role of PKB/Akt, Erk1/2 and p38MAPK signaling pathways on GPC3 induced susceptibility to cell death, 10^5 cells were seeded in triplicate in 96-well plates, in 200 μl of complete medium supplemented with 5% FCS. After 24 h, cells were washed with PBS and cultured in the absence of FCS for 24 additional hours. Monolayers were then treated with the pharmacological inhibitors LY294002 (5, 10 and 15 μM), UO126 (2.5, 5 and 10 μM) and SB202190 (5, 10 and 15 μM) or with the vehicle alone (DMSO). At 48 h after treatment, viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product as calculated by the 492/620 nm absorbance ratio (Cell Titer 96 TM, Promega Corp).

Transient transfections

Gene reporter assays

To analyze the effects of GPC3 on NFκB transcriptional activity, 7×10^4 cells/well were seeded in a 24-well plate and cotransfected with a firefly-luciferase reporter vector containing κB response elements (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 (Dual-Luciferase Reporter Assay, Promega).

Dominant negative mutant of p38 (DNp38)

To determine the role of p38 in GPC3 modulated apoptosis, cells growing on 35 mm dishes were transfected with the dominant negative mutant of this molecule or the appropriate empty vector (genteelly offered by Dr. Silvio

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Gutkind) using FuGene (Roche). As transfection control, protein extracts were obtained to evaluate p38 expression level by Western blot. After 24 h, transfected cells were seeded on 96-well plates and depleted of serum for 48 additional hours. Cell viability was evaluated by MTS.

Western blot

For the preparation of total protein extracts, subconfluent monolayers growing in 100 mm plates were depleted or not of FCS for 24 and 48 h, or stimulated with serum pulses for 30 and 60 min, or treated overnight with SB202190 (15 μM). Then, cells were 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 [23]. All samples were resuspended 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 analyzed by Western blotting using the following primary antibodies: anti-cleaved Caspase-3 (Cell Signaling), anti-Caspase 9 p35 (H-170, Santa Cruz Biotech), anti-Bax, anti-Bcl-2, anti Bcl-xL, anti-Bad (all from Becton Dickinson, PharMingen), anti-Cylin D1, anti-Cyclin B1, anti-p16, anti-p21, anti-p27 (all from Santa Cruz Biotech), anti-phospho-Erk (Tyr 204), anti-phospho-Akt (Ser 473), anti-phospho-p38 (Thr 180/Tyr 182) (all from Cell Signaling). Then membranes were incubated with anti-mouse, anti-rat or anti-rabbit horseradish peroxidase-conjugated antibodies (Sigma) as corresponded. Protein bands were detected using the ECL Reagent (Amersham Biosciences). Electrophoretic band images were analyzed by densitometry and expressed in optical density arbitrary units (OD) (Molecular AnalystTM GS-700, BioRad). Total Erk, Akt and p38 levels were determined by stripping and reproving the membranes with anti-Erk, anti-Akt and anti-p38 antibodies (Cell Signaling).

Immunocytochemistry

For immunocytochemistry cell clones were grown on glass coverslides. At 48 h cultured cells were washed twice with ice cold PBS and fixed with buffered-formaldehyde 3.7% at 4°C for 10 min. Then, cells were treated with H_2O_2 10% in methanol for 15 min to eliminate endogenous peroxidase and blocked for 1 h with 2.5% skim milk in PBS. Primary anti-cyclin D1, anti-Cyclin B1, anti-p16, anti-p21 and anti-p27 antibodies diluted in PBS (1:500) were incubated overnight at 4°C. After washing with PBS, coverslides were incubated with biotinylated anti-mouse or anti-rabbit and streptavidin-peroxidase conjugate (Vector). The immunoreactive product was revealed with a substrate solution of 3-3′ diaminobenzidine (DAB).

PCR analysis

Total RNA extraction and cDNA synthesis

Total RNA was prepared from LM3-GPC3 and LM3-vector cells growing in the presence or absence of FCS, or treated with the pharmacological inhibitor SB202190 (15 μM), 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 the reverse transcriptase enzyme.

Quantitative real time PCR

The expression levels of selected genes were determined by Quantitative real time PCR (qPCR) analysis. Primers were designed spanning an intron within the cDNA sequence target, making the cDNA amplification product easily distinguishable from the genomic product. These primers that amplified a 100–150 bp length amplicons, with a melting temperature of 60°C, were synthesized by Invitrogen. Analysis of dissociation curves from each PCR product, demonstrated a single peak for the whole set of primers. Primer sequences were as follows (5′–3′):

p21

F-GTCTGAGCGGCCTGAAGATTC
R-TTCAGGGTTTTCTCTTGCAGAAG

p53

F-CGACCTATCCTTACCATCATCACA
R-CACAAACACGAACCTCAAAGCT

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Bax

F-CAAGAAGCTGAGCGAGTGTC
R-GAAGTTGCCGTCTGCAAACA

Bcl-xL

F-CAGACCCAGTGAGTGAGCAG
R-CCGGTTGCTCTGAGACATTT

PUMA

F-GCGGAGACAAGAAGAGCAAC
R-TCCAGGATCCCTGGGTAAG

XIAP

F-GGCCAGACTATGCCCATTTA
R-CCACCACAACAAAAGCATTG

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 cDNA, 12.5 µl 2× 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 7500 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 housekeeping genes (tubulin, actin, HPRT, 36B4, GAPDH). For quantitative results the levels of each transcript were normalized to the level of internal housekeeping HPRT gene since its expression showed the less variability among clones. Results were expressed as fold-change using the ΔCt method [24].

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. Differences among groups were calculated by applying ANOVA/Bonferroni's tests as indicated. A value of $P < 0.05$ was considered to be significant.

Results

GPC3 reexpression induces cell death and modulates apoptotic and cell cycle regulatory molecules

Evidences presented by other authors [8] and our own results [12] suggest that GPC3 is involved in the control of mammary cell survival. Taking this into account, first we studied the survival capacity of LM3-GPC3 and LM3-vector clones in response to nutrient depletion employing Annexin-V staining and flow cytometry, an accepted methodology for an apoptosis assessment. As shown in Fig. 1A, while at 48 h of starving only about 18% apoptotic LM3-vector cells were detected, about 32% apoptotic LM3-GPC3 cells were recorded in the same period of time. In addition, this difference became greater if we compare early apoptotic cells (9% in LM3-vector vs. 23% in LM3-GPC3). Figure 1 also shows that after 96 h of nutrient depletion approximately 37% of LM3-vector cells were apoptotic, in contrast with 63% of LM3-GPC3 ones. It is interesting to note that the percentage of serum starving induced apoptosis found in LM3-vector cells at 96 h was similar to those found in LM3-GPC3 clones at 48 h. In other words, GPC3 reexpression sensitized LM3 cells, which needed a shorter time of stress stimuli to die.

Since GPC3 expressing and control clones showed different apoptotic death susceptibility, we studied the expression of some molecules involved in the apoptotic pathway. We analyzed by means of WB and/or qPCR, the expression of pro-apoptotic p53, Bax, PUMA, Caspase-3 and Caspase-9, and the anti-apoptotic Bcl-2, Bcl-xL and XIAP molecules.

We determined that p53 mRNA levels were higher in LM3-GPC3 clones, both in the presence or absence of serum (Fig. 1B). In association, the levels of Bax and PUMA were significantly enhanced in GPC3-expressing clones (Fig. 1C, D). GPC3 reexpression also induced a significant increase in the activity of Caspase-9 and Caspase-3, the main initiator and effector apoptosis molecules, respectively (Fig. 1F, G).

Fig. 1 Effect of GPC3 reexpression on LM3 cells susceptibility to cell death and on apoptotic control molecules. Subconfluent monolayers depleted of FCS for 48 or 96 h were stained with Annexin-V+ FITC plus PI and examined by flow cytometer. **A** *Flow cytometry pictographs*: viable cell population (Annexin-V−/PI−) in Sect. 3; early apoptotic cell population (Annexin-V+/PI−) in Sect. 4; late apoptotic cell population in Sect. 2 (Annexin-V+/PI+) and necrotic cell population in Sect. 1 (Annexin-V−/PI+); *Bars graph*: *bars* represent the mean ± SD of the percentage of apoptotic cells (early + late). *a* $P < 0.001$ vs. all the rest; *b* $P < 0.01$ vs. LM3-vector 48 h treatment; ANOVA/Bonferroni's tests. *qPCR*: Total RNA from cells grown in the presence or absence of FCS for 48 h, were analyzed by qPCR. The level of each transcript was normalized to the expression level of internal housekeeping control HPRT gene. *Bars* represent the mean ± SD of the following rates: p53/HPRT (**B**), Bax/HPRT (**C**), PUMA/HPRT (**D**), XIAP/HPRT (**E**), Bcl-xL/HPRT (**H**), Bax/Bcl-xL (**I**) ratios. The results are expressed as fold of LM3-vector relative gene expression. *a* $P < 0.001$, *b* $P < 0.01$; *c* $P < 0.05$ vs. LM3-vector; ANOVA/Bonferroni's tests. *WB*: Total protein extracts obtained from cells grown in the presence of FCS were analyzed using anti Bax (**C**), anti active Caspase-9 (**F**) and Caspase-3 (**G**), anti-Bcl-xL (**H**) and anti-Bcl-2 (**J**) antibodies. Loading was standardized by actin levels. *Bars* represent the mean ± SD of the molecules/actin levels in OD arbitrary units. *a* $P < 0.001$ vs. LM3-vector; ANOVA/Bonferroni's tests

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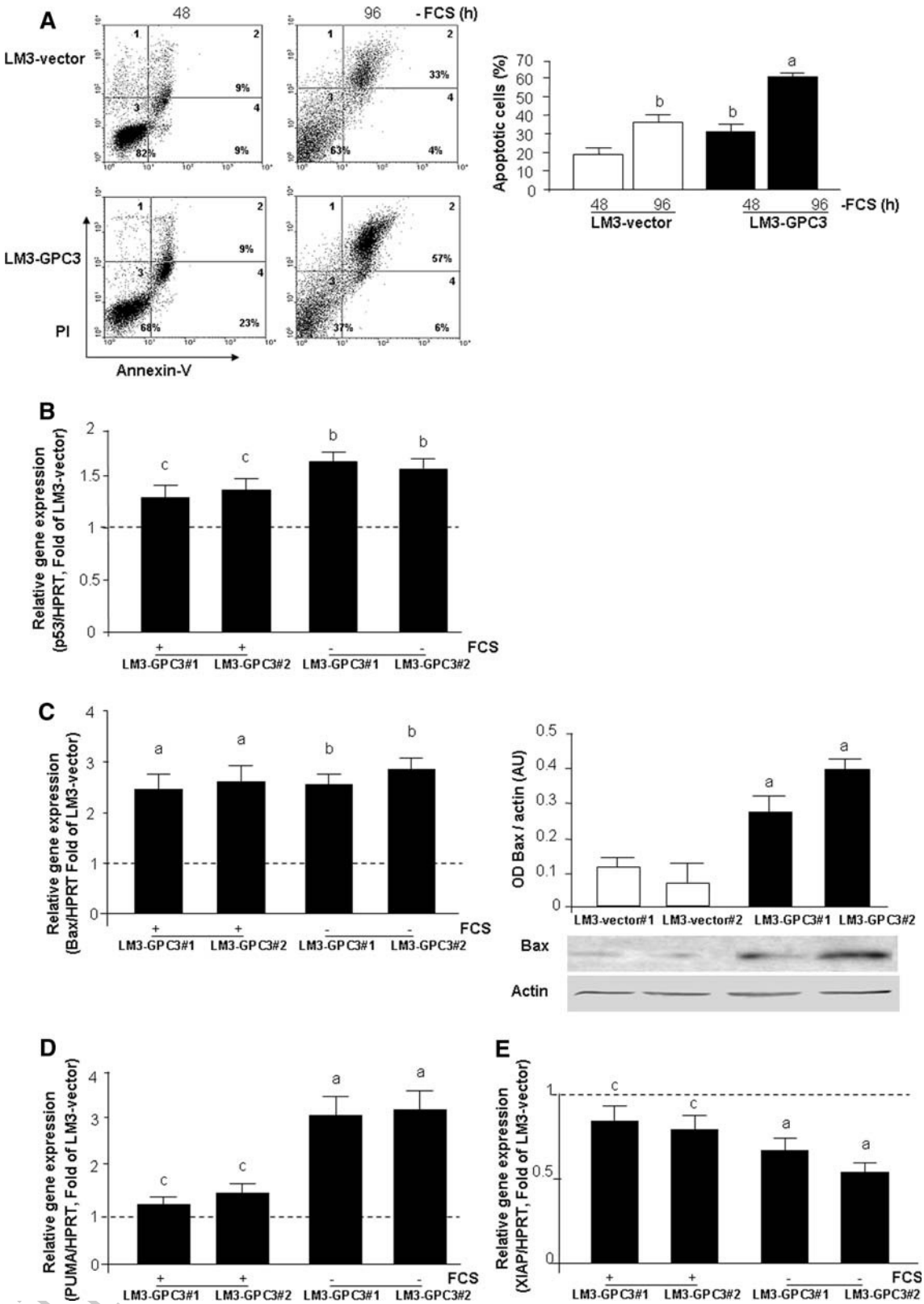

On the other hand, LM3-GPC3 clones presented a reduction in the expression of Bcl-xL and Bcl-2 (Fig. 1H, J). In agreement with the higher apoptosis level shown, the ratio Bax/Bcl-xL was elevated in LM3-GPC3 cells (Fig. 1I). Finally, mRNA levels of XIAP (inhibitor of Caspase 3, 7 and 9) were significantly reduced in LM3-GPC3 cells (Fig. 1E).

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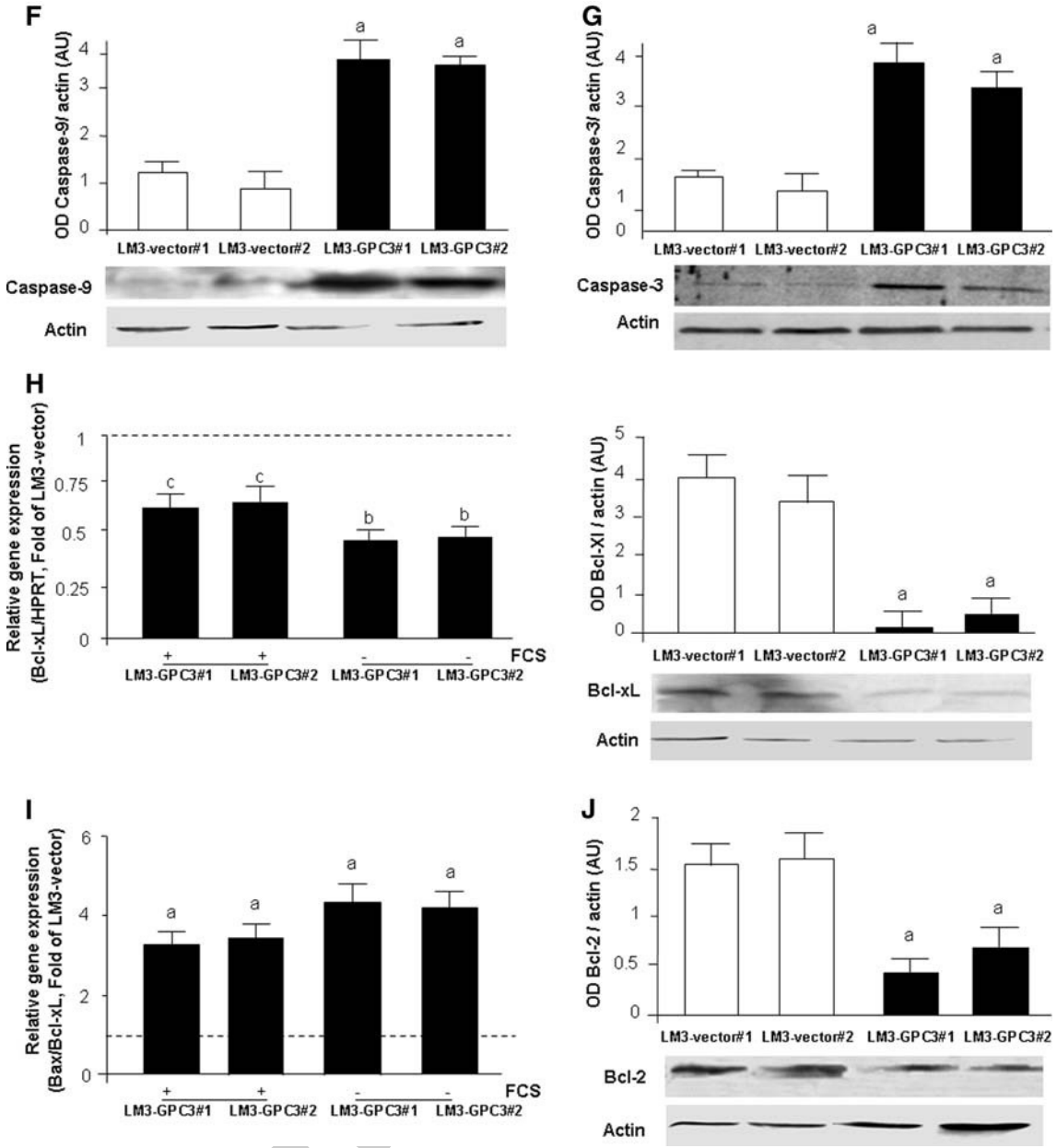

Fig. 1 continued

Next, we studied the cell cycle machinery employing qPCR, WB and ICC. We found higher expression levels of the CDKI p21 in GPC3 expressing cells (Fig. 2A). Similar results were obtained for p27, another member of p21 family (Fig. 2B). In addition, ICC demonstrated that both CDKIs were mainly localized in the nuclei of LM3-GPC3 cells (Fig. 2A, B). On the other hand, GPC3 did not modulate either the basal expression of Cyclins D1 and B1, or that of the CDKI p16 (Fig. 1C–E).

Fig. 2 Effect of GPC3 reexpression on cell cycle regulatory molecules. *qPCR* (**A**): Total RNA from clones grown in the presence or absence of FCS for 48 h was retrotranscripted and analyzed by qPCR reactions. The level of p21 transcript was normalized to the expression level of the internal housekeeping HPRT gene. *Bars* represent the mean ± SD of p21/HPRT ratio and are expressed as fold of LM3-vector relative p21 expression. *b* $P < 0.01$ and *c* $P < 0.05$ vs. LM3-vector; ANOVA/Bonferroni's tests. *WB and ICC*: LM3-GPC3 and LM3-vector cells, growing in presence of FCS, were analyzed by WB and ICC as it was indicated in "Materials and methods". The expression of each antigen was quantified and represented as histograms, where: (**A**) p21, (**B**) p27, (**C**) p16, (**D**) Cyclin D1 and (**E**) Cyclin B1. Actin was always included as a control of seeding. *Bars* represent the mean ± SD. *b* $P < 0.01$ vs. LM3-vector; ANOVA/Bonferroni's tests

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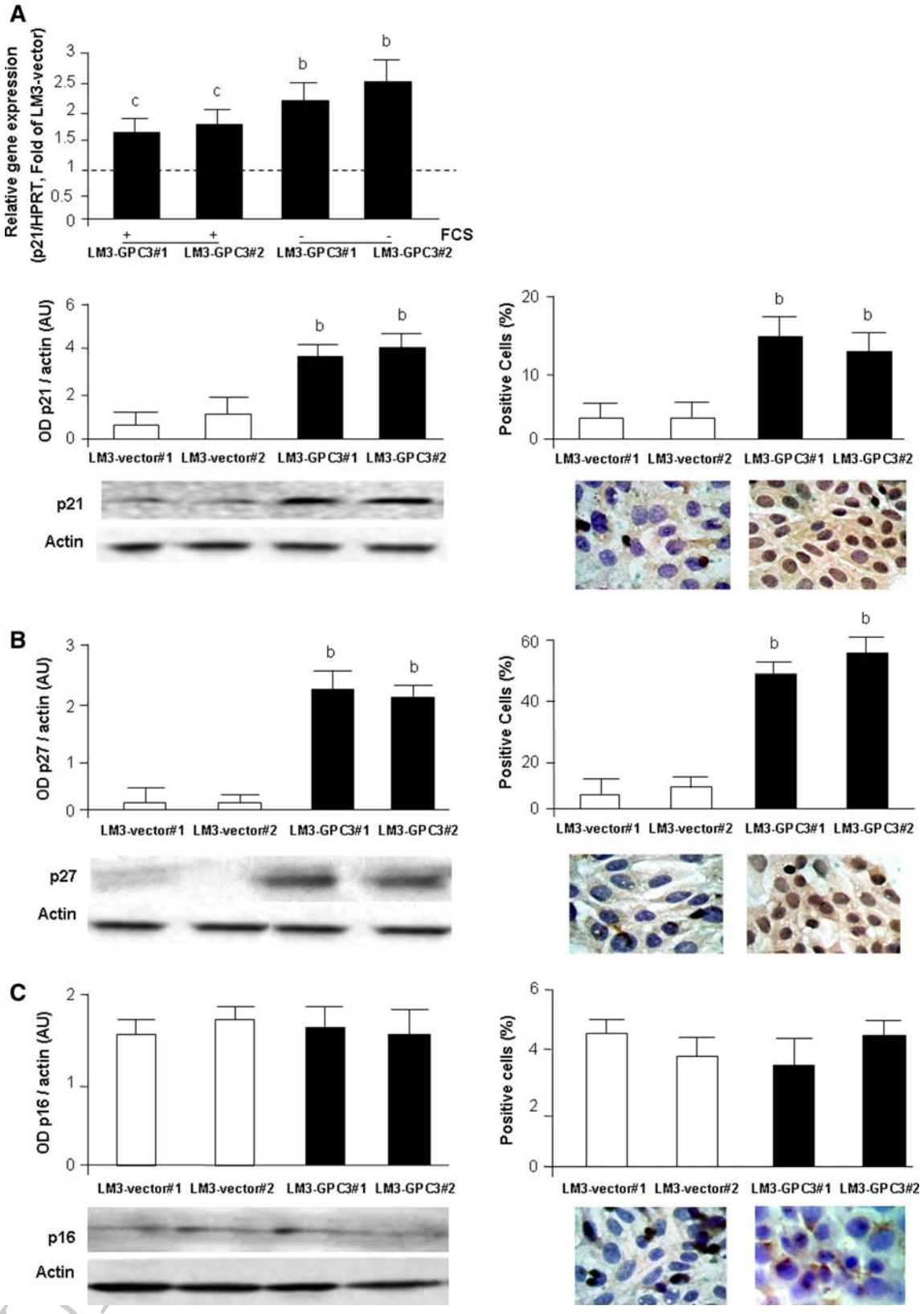

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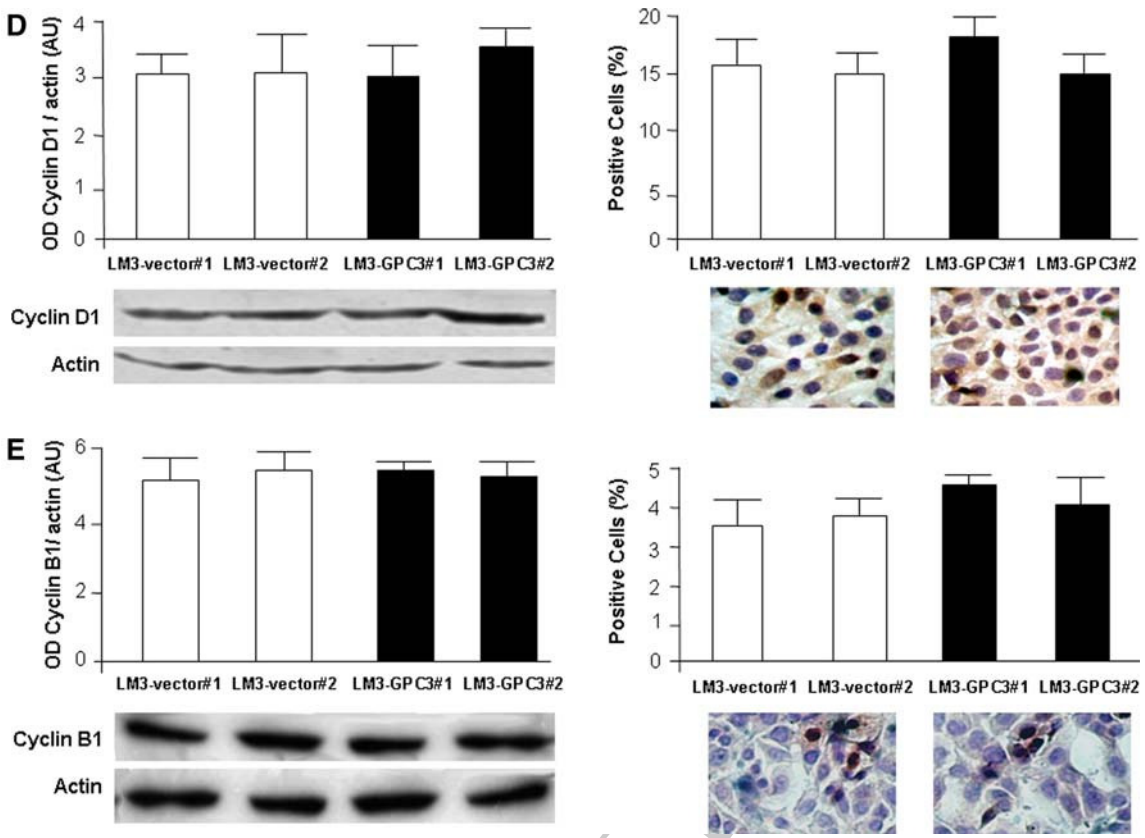

Fig. 2 continued

GPC3 reexpression modulates neither Erk1/2MAPK nor NFκB signaling pathways

Next, we studied whether Erk1/2MAPK and NFκB signaling pathways, which have important effects on proliferation and cell survival, were modulated by GPC3 reexpression.

The activation of the pro-mitogenic Erk1/2MAPK pathway was analyzed checking the levels of phospho-Erk by WB. As shown in Fig. 3A, no differences were found among GPC3 and control clones, either in basal conditions or after serum stimulation. Moreover, the inhibition of the ErkMAPK pathway with the MEK1/2 pharmacological inhibitor UO126 induced a similar diminution in the viability of both GPC3 and vector clones and was not able to revert the GPC3 induced apoptosis susceptibility (Fig. 3B).

On the other hand, the pro-survival NFκB pathway was analyzed employing a gene reporter assay. Cells were transfected with a vector containing the response elements κb upstream the luciferase gene. As shown in Fig. 3C, NFκB activity was not affected by GPC3 reexpression (Fig. 5).

Altogether, these results strongly suggest that the effect of GPC3 on LM3 mammary tumor cells death susceptibility would not be dependent on the modulation of Erk1/2MAPK or NFκB signaling pathways.

GPC3 reexpression induces an inhibition of the pro-survival Akt signaling pathway

Looking for possible effectors of GPC3 action on cell death, next we studied the activation of Akt, a kinase able to promote survival blocking apoptosis [25]. We found that whereas this pathway was constitutively active in control cells even in serum withdrawal conditions, GPC3 reexpression induced a four to fivefold decrease of phospho-Akt basal levels. In addition, although LM3-GPC3 cells responded to mitogenic signals by increasing 3–4 times Akt phosphorylation, this enhancement did not even reach the phospho-Akt levels found in LM3-vector cells (Fig. 4A).

With the aim of confirming Akt participation in the survival of LM3 cells, subconfluent monolayers were incubated with an Akt pathway inhibitor for 48 h. This treatment induced a remarkable decrease of LM3-vector cells viability, reaching similar levels to those of LM3-GPC3 clones without treatment (Fig. 4B). These results

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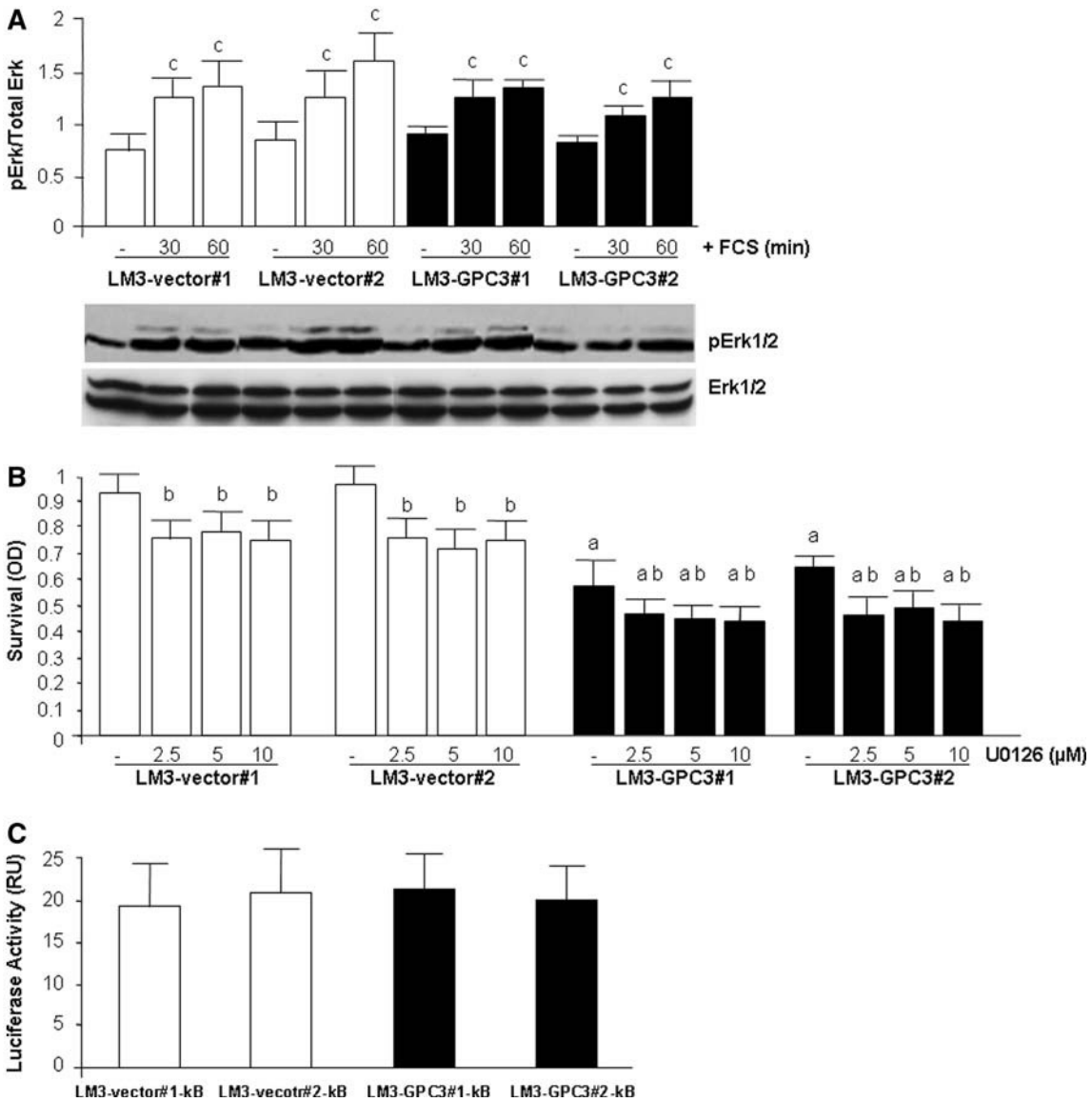

Fig. 3 Effect of GPC3 reexpression on Erk1/2MAPK and NKκB signaling pathways. **A** Protein extracts from GPC3-transfectans and control clones starved or pulsed with FCS (30 or 60 min) were subjected to WB for total and phospho-Erk1/2. *Bars* represent the mean ± SD of phospho-Erk/Total Erk ratio levels. *c* $P < 0.05$ vs. corresponding control; ANOVA/Bonferroni's tests. **B** Subconfluent monolayers were simultaneously depleted of FCS and treated with UO126 or with DMSO during 48 h. Viability was assessed by MTS by determining the absorbance 492/620 nm (OD). *Bars* represent the mean ± SD of OD values. *a* $P < 0.001$ vs. LM3-vector (treated or not); *b* $P < 0.01$ vs. its corresponding control; ANOVA/Bonferroni's tests. **C** LM3-GPC3 and LM3-vector cells were cotransfected with a firefly-luciferase reporter vector containing κB response elements and with a renilla-luciferase vector. *Bars* represent the mean ± SD of luciferase activity. The difference among clones was not statistically significant by ANOVA test

emphasize the relevance of an active Akt pathway in the survival of LM3 cells.

GPC3 reexpression induces an activation of the pro-apoptotic p38MAPK signaling pathway

Next, we analyzed the activation of p38MAPK pathway, by studying the levels of p38 phosphorylation in cells growing both in presence or absence of serum. Whereas LM3-vector cells were not able to activate p38 even after serum depletion, GPC3 reexpression enhanced about four times the basal levels of phospho-p38, which raised up to ten times after serum starvation (Fig. 5A).

To confirm the role of p38 signaling pathway in GPC3 induced LM3 cells death, we evaluated cells viability after transient transfection with a DNp38. The overexpression of the above mentioned protein was confirmed by WB (Fig. 5B, inset) and the viability was evaluated by MTS.

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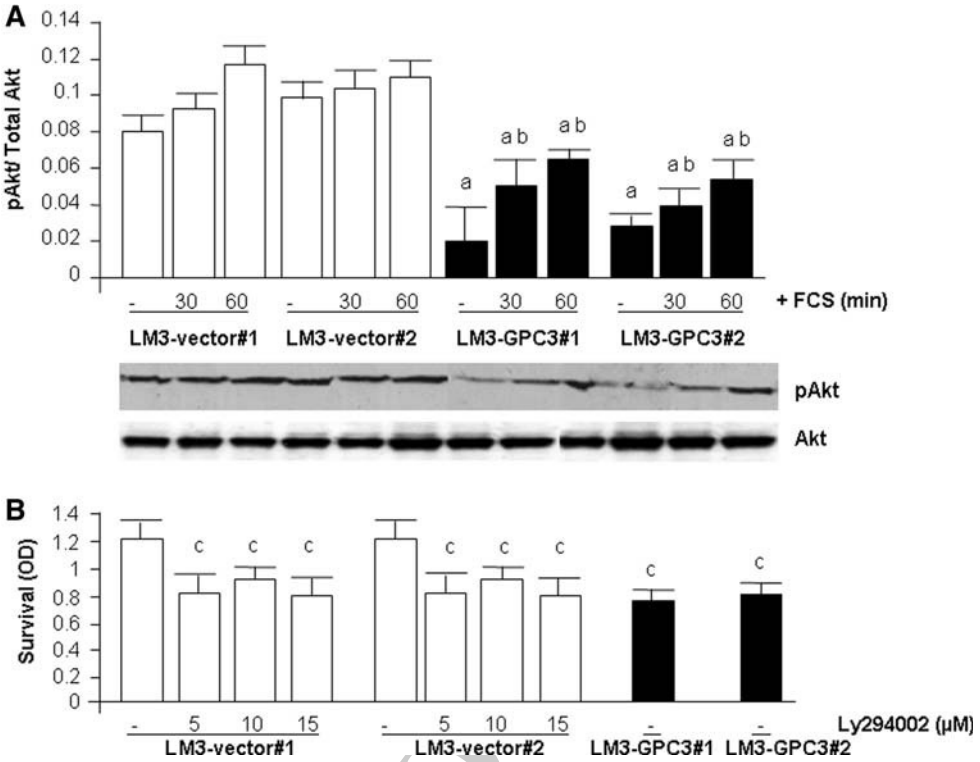

Fig. 4 Effect of GPC3 reexpression on PKB/Akt signaling pathway. **A** Protein extracts from LM3-GPC3 and LM3-vector clones starved or pulsed with FCS (30 or 60 min) were resolved in WB for total and phospho-Akt. *Bars* represent the mean ± SD of phospho-Akt/Total Akt levels. *a* $P < 0.001$ vs. LM3-vector; *b* $P < 0.01$ vs. starved LM3-GPC3; ANOVA/Bonferroni's tests. **B** Subconfluent monolayers were simultaneously depleted of FCS and treated with LY294002 or with the vehicle during 48 h. Viability was assessed through MTS. *Bars* represent the mean ± SD of OD values. *c* $P < 0.05$ vs. untreated LM3-vector; ANOVA/Bonferroni's tests

We found that the expression of DNp38 mutant increased LM3-GPC3 cell survival in serum-free conditions, reaching similar levels to those of LM3-vector cells (Fig. 5B).

To further support the hypothesis that GPC3 promotes mammary cells death trough the modulation of p38 pathway, subconfluent monolayers were treated with a pharmacological specific inhibitor of this MAPK pathway (SB202190). As shown in Fig. 5C, this treatment clearly reverted the decrease of cell viability induced by serum depletion in LM3-GPC3 cells. Additionally, we confirmed employing flow cytometry that upon p38MAPK pathway inhibition GPC3 was not able to induce apoptosis (Fig. 5D). Density plots shows a significant reduction in the GPC3-induced susceptibility to apoptosis when p38 pathway is inhibited. After 96 h of starvation about 30% of apoptotic LM3-GPC3 cells were detected, but when they were simultaneously treated with SB202190 the level of apoptotic cells decreased up to only 5%.

Altogether, these results strongly suggest that GPC3 may be regulating cell survival and/or death through the activation of p38MAPK pathway.

Finally, we also evaluated whether the expression of apoptosis control molecules modulated by GPC3 was altered when cells are treated with the p38MAPK inhibitor. We found that the inhibition of p38 induced an increase, at protein and/or mRNA level, in the anti-apoptotic molecules Bcl-2, Bcl-xL and XIAP (Fig. 6A–C). In contrast, LM3-GPC3 cells treated with SB202190 presented lower levels of the pro-apoptotic molecules Bax and PUMA (Fig. 6D, E).

Fig. 5 Effect of GPC3 reexpression on p38MAPK signaling pathway. **A** Protein extracts from LM3-GPC3 and LM3-vector clones grown in the presence or absence of FCS (24 or 48 h) were analyzed by WB for total and phospho-p38. *Bars* represent the mean ± SD of phospho-p38/Total p38 levels. *a* $P < 0.001$ vs. all the others; *c* $P < 0.05$ vs. LM3-vector -FCS; ANOVA/Bonferroni's tests. **B** Cells were transfected with a DNp38 mutant. Protein extracts were evaluated for p38 expression level by WB (inset). Transfected cells were depleted of serum (48 h) and cell viability was evaluated by MTS. *Bars* represent the mean ± SD of OD values *b* $P < 0.01$ vs. all the rest; ANOVA/Bonferroni's tests. **C** Subconfluent monolayers were treated with SB202190, or with the vehicle (DMSO). Viability was assessed by MTS, by determining the absorbance 492/620 nm (OD). *Bars* represent the mean ± SD of OD values. *a* $P < 0.001$ vs. LM3-vector; *b* $P < 0.01$ vs. treated LM3-GPC3; ANOVA/Bonferroni's tests. **D** Subconfluent LM3-GPC3 monolayers depleted of FCS (96 h) were simultaneously treated with 15 μM. Then, cells were stained with Annexin-V+ FITC plus PI and examined by flow cytometer. *Flow cytometry pictographs*: viable cell population (Annexin-V−/PI−) in Sect. 3; early apoptotic cell population (Annexin-V+/PI−) in Sect. 4; late apoptotic cell population in Sect. 2 (Annexin-V+/PI+) and necrotic cell population in Sect. 1 (Annexin-V−/PI +); *Bars graph*: *bars* represent the mean ± SD of the percentage of apoptotic cells (early + late). *a* $P < 0.001$ vs. not treated LM3-GPC3; ANOVA/Bonferroni's tests

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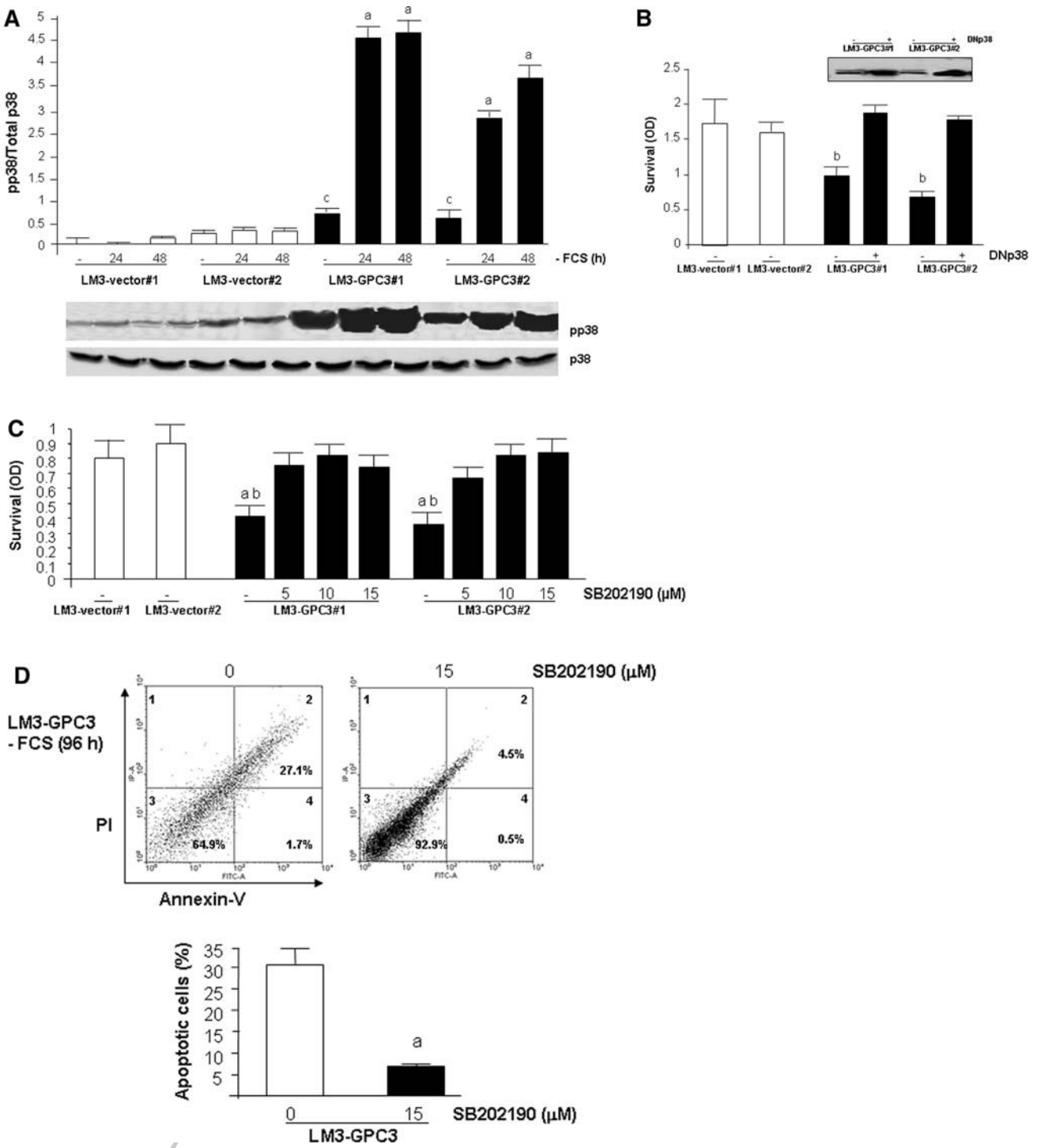

Discussion

Since GPC3 is closely linked to tissue growth and development, it is not surprising that alterations in its function are associated with tumor pathologies. The loss of GPC3 expression seems to be a key event in the malignant progression of mammary tumors [12, 26]. By means of an exhaustive in vivo and in vitro analysis of two GPC3 reexpressing clones, we previously determined that this proteoglycan would be acting as a metastatic suppressor [12]. Since metastatic dissemination is the event that darkens the prognosis of the oncologic patient, the minutiae study of molecular triggers involved in the metastatic process is elemental for the mammary cancer therapy [27].

Hanahan and Weinberg [28] have proposed some common characteristics to all the malignant cells, being

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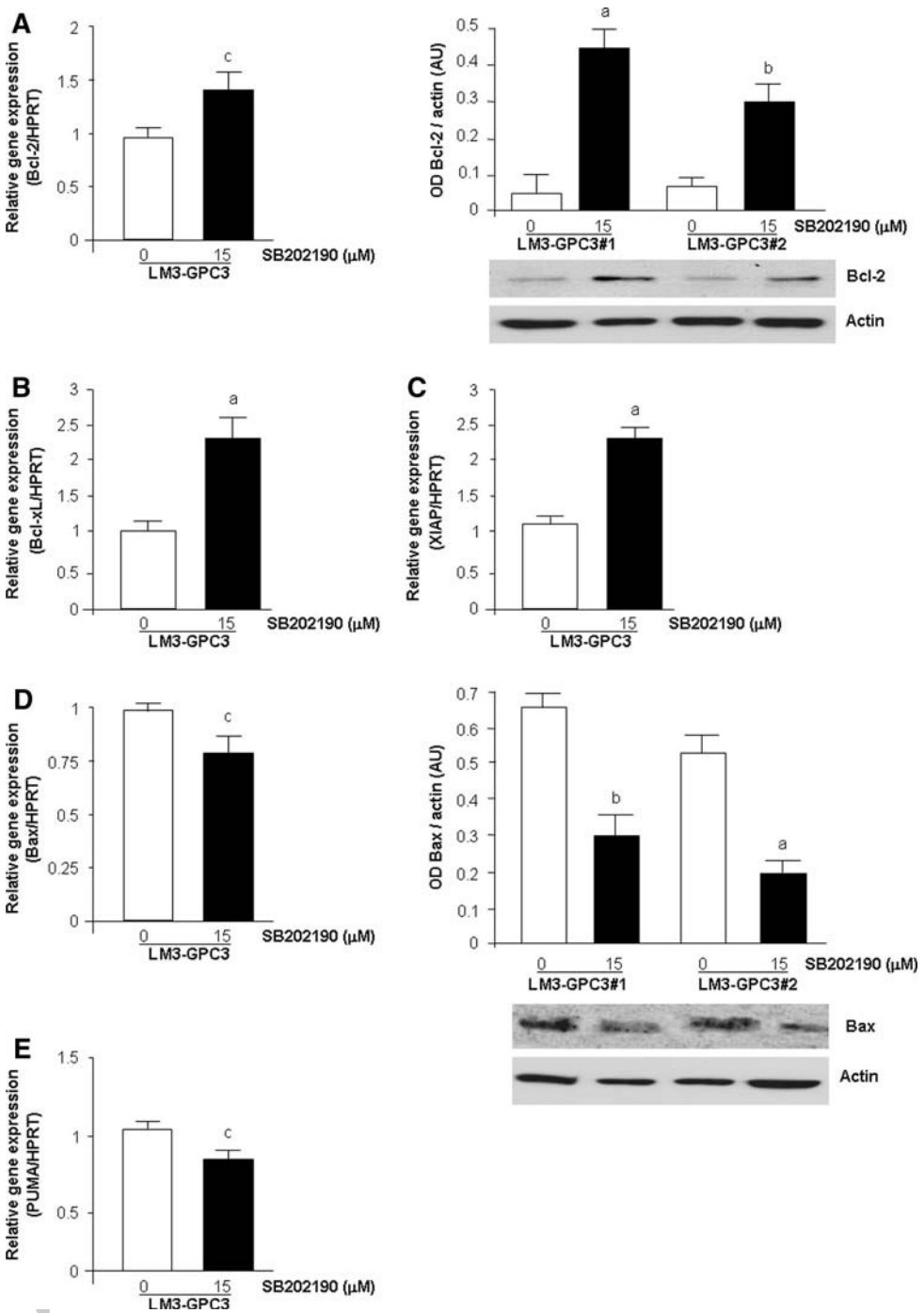

Fig. 6 Effect of p38MAPK pathway inhibition on GPC3 modulated apoptotic molecules expression. *qPCR*: Total RNA from LM3-GPC3 clones depleted of FCS and treated ON with SB 202190 (15 μM), was retrotranscripted and analyzed by qPCR reactions. The level of transcripts was normalized to the expression level of the internal housekeeping HPRT gene. *Bars* represent the mean ± SD of Bcl-2/HPRT (**A**), Bcl-xL/HPRT (**B**), XIAP/HPRT (**C**), Bax/HPRT (**D**) and PUMA/HPRT (**E**) ratios. *b* $P < 0.01$ and *c* $P < 0.05$ vs. not treated LM3-GPC3; ANOVA/Bonferroni's tests. *WB*: LM3-GPC3 cells treated ON with SB 202190 (15 μM), were analyzed by WB as it was indicated in "Materials and methods". The expression of each antigen was quantified and represented as histograms, where: (**A**) Bcl-2 and (**D**) Bax. Actin was always included as a control of seeding. *Bars* represent the mean ± SD. *a* $P < 0.001$ and *b* $P < 0.01$ vs. not treated LM3-GPC3; ANOVA/Bonferroni's tests

resistance to apoptosis the most significant of them. So, in the present work we studied the effect of GPC3 reexpression on stress induced LM3 cell death, as well as the possible signaling pathways involved in this event. We demonstrated that GPC3 reexpression in tumor mammary cells increases the susceptibility to serum withdrawal induced apoptosis. In other words, GPC3 reexpression induced a decrease of the resistance to apoptosis acquired by LM3 cells. Our results are in agreement with those reported for the human mammary cell line MCF7, where GPC3 transfection generated smaller number of clones than the transfection with an inactive GPC3 mutant [26]. In addition, the role of GPC3 in apoptosis was also suggested by the phenotype of patients with the overgrowth Syndrome of Simpson-Golabi-Behmel, associated with loss-of-functions mutations in the GPC3 gene [29].

Inside the apoptotic machinery, the p53 tumor-suppressor protein acts inhibiting the growth of stressed or

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abnormal cells, and so prevents cancer development [30]. In agreement with the higher apoptotic rate, we determined that GPC3 reexpression induced an increase of p53 mRNA levels in LM3 cells. To our knowledge, this is the first report indicating that GPC3 is able to modulate p53 expression.

It is known that p53 regulates apoptosis through both transcriptional-dependent and -independent mechanisms. Thus, p53 functions as a transactivator to upregulate downstream pro-apoptotic genes (e.g. Bax, Noxa, and PUMA), and as a repressor downregulating anti-apoptotic ones (e.g. Bcl-2), promoting apoptosis [31]. In agreement with the higher levels of p53 detected in LM3-GPC3 cells, we found a concomitant increase of the pro-apoptotic Bax and PUMA genes and a decrease of the anti-apoptotic Bcl-2 gene. Besides, another member of the Bcl-2 family, the anti-apoptotic Bcl-xL protein, was also downregulated in GPC3 expressing cells. Additionally, GPC3 reexpression induced a significant increase in the activity of Casaspe-9 and -3. On the other hand, we found by qPCR that LM3-GPC3 cells express less caspase inhibitor XIAP, which in turn might facilitate caspase activation.

In sum, the inhibition of apoptosis resistance detected in LM3-GPC3 cells could be explain, at least partly, by an increase in the expression and/or activity of the pro-apoptotic molecules p53, Bax, PUMA, Caspase-9 and Caspase-3 and the decrease of the anti-apoptotic Bcl-2, Bcl-xL and XIAP. Though other authors have associated GPC3 with apoptosis, this work is the first giving evidences of molecular basis by which GPC3 would be acting on this important cell property. On the other hand, identifying p53 modulators and understanding the mechanisms through which they alter the p53-determined cell fate has important therapeutic implications, especially if these putative molecules could be manipulated to promote apoptosis in tumors.

It is known that cancer cells are able to evade antiproliferative signals by means of alterations in the cell cycle control molecules [28]. Among them is the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, which induction by p53 is central to the cell cycle arrest [30]. In this work, and in association with the higher expression of p53 detected in LM3-GPC3 clones, we found enhanced mRNA and protein levels of p21. Besides, the levels of p27, another CDKI of the p21 family, were also increased in LM3-GPC3 cells. Moreover, by means of ICC we observed that GPC3 expressing clones showed a higher staining of these CDKIs at nuclear level, suggesting its elevated biological functionality as checkpoint arrest molecules.

Among the typical signaling pathways usually associated with proliferation and apoptosis inhibition, we found that ErkMAPK and NFκB were not modulated by GPC3. ErkMAPK pathway transduces mitogenic and anti-apoptotic signals and is altered in many human and experimental tumors [32]. Our results indicated that this pathway would not be involved in GPC3 signaling since neither phospho-Erk levels nor cell viability after specific inhibitor treatment was affected by GPC3 reexpression. On the other hand, NFκB transcription factor has an important role in cell development, survival and oncogenesis, activating anti-apoptotic genes expression [33]. We found that GPC3 is not able to modulate the activity of this transcription factor.

Akt kinase has been extensively studied since an increase in its activity provides tumor cells with an improvement of their survival, by inhibition of apoptosis [34]. In contrast to Song et al. [17], who reported that GPC3 does not modulate Akt, we found that GPC3 reexpression prevented the Akt overactivation found in our model of mammary tumor cells. Furthermore, GPC3 reexpression conferred LM3 cells the capacity to phosphorylate Akt after an exogenous mitogenic stimulus. In addition, control LM3-vector cells always presented saturated levels of phospho-Akt both in the presence or absence of FCS, but the treatment with an Akt specific inhibitor diminished their viability, which reached similar survival values as LM3-GPC3 cells did. These results indicate that Akt is an important pathway for the survival of these mammary tumor cells. However, additional experiments are necessary to demonstrate the real role of Akt in GPC3 apoptosis promotion.

Since p38MAPK signaling pathway is able to induce apoptosis [35], we studied whether GPC3 reexpression could modulate phospho-p38 levels. We demonstrated that p38MAPK activation is critical for GPC3 promotion of cell death. When p38MPAK pathway was blocked or inhibited, GPC3 was unable to induce apoptosis. We also established that the activation of p38MAPK mediates GPC3 modulation of the expression of Bcl-2, Bcl-xL, XIAP, Bax and PUMA. When p38MAPK signaling was inhibited, GPC3 was not able to induce up-regulation of the pro-apoptotic molecules Bax and PUMA, nor could it reduce the levels of the anti-apoptotic Bcl-xL, Bcl-2 and XIAP. The role of p38MAPK pathway in the regulation of apoptotic molecules was previously reported by other authors [36, 37], however, this is the first report that demonstrates that GPC3 induces an increase in apoptosis susceptibility of mammary tumor cells through p38MAPK signaling pathway activation and the consequent regulation of Bax, PUMA, Bcl-xL, Bcl-2 and XIAP expression.

In sum, our results provide new evidences supporting the idea that GPC3 regulates cell survival and apoptosis, possibly activating p38MAPK and inhibiting PKB/Akt pathways. Our previous studies [12, 18] together with the present ones make us propose that GPC3 induces alterations towards a less aggressive phenotype in mammary cancer cells due to a normalization of diverse parameters. Even though the hierarchical order by which GPC3 may

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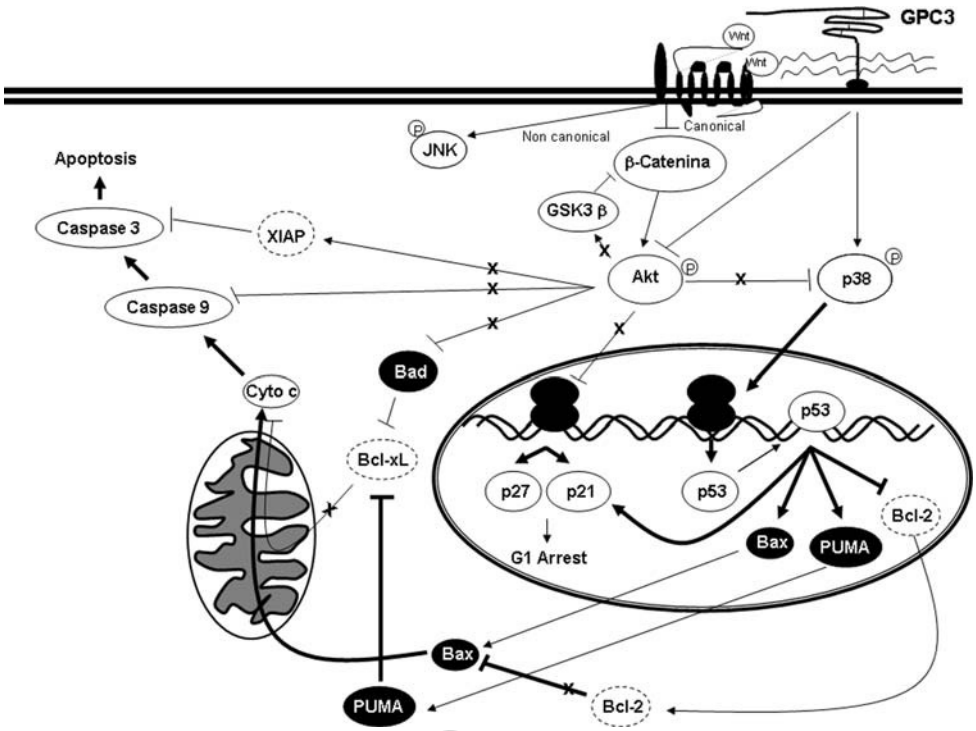

Fig. 7 Hypothetical model of GPC3 signaling interactions in LM3 cells. GPC3 may regulate cell survival and apoptosis, possibly activating p38MAPK and inhibiting PKB/Akt pathways. Even though the hierarchical order by which GPC3 may alter the different pathways is difficult to establish, we postulate a suppose model. We entrust that future experiments will give us the possibility to discover the exact GPC3 signaling pathway. We believe that our work is clinically relevant since the knowledge of the molecular mechanisms through which GPC3 supports the homeostasis of normal mammary cells has fundamental relevance both to understand the breast cancer biology and to find a molecular targeted breast cancer therapy

alter the different pathways is difficult to establish due to the multifunctionality of this protein and to the crosstalk among pathways, we postulate a hypothetical model depicted in Fig. 7.

It has been described that glypicans might work as co-receptors of Wnt factors [38]. Our previous results, recently published, show that GPC3 is able to activate the non canonical Wnt signaling in LM3 cells, probably through the interaction with Wnt receptors or their ligands [18]. We postulate that the enhancement of the non canonical Wnt activity, in turn, would induce an inhibition of the canonical one. Nevertheless, we can not discard that GPC3 negatively regulates, in a direct way, the canonical Wnt pathway. From this Wnt signaling modulation many responses and interactions would be generated, being Akt a fundamental mediator. Now we demonstrate that GPC3 is able to reduce the Akt overactivation detected in LM3 cells. As no direct relationship has been demonstrated between GPC3 and Akt, it is probable that intermediate molecules are acting. Among them, it is reported that the Akt inhibitor PTEN is able to form complexes with members of non canonical Wnt signaling [39]. Another interaction between both pathways could be mediated by β-Catenin/RAS union [40].

We also demonstrate that the apoptosis permissive scenario induced by GPC3 could be due by the activation of p38MAPK. Since it has been described that Akt inhibits p38MAPK [25], the diminished levels of phospho-Akt could be promoting p38MAPK activation. On the other hand, the tumor suppressor p53 is a transcriptional target of p38MAPK pathway [41]. So, the elevated levels of p38MPAK activity would be responsible for the higher p53 mRNA expression detected in LM3-GPC3 clones and the concomitant modulation of several apoptosis and cell cycle regulatory molecules. In addition, it is known that Akt signaling blocks apoptosis by inhibiting Caspase-9. So, it is probable that the decreased phospho-Akt levels present in LM3-GPC3 cells may also be responsible for the increased Caspase-9 activity. This cysteine proteinase caspase, in turn, could induce a higher cleavage of the effector Caspase-3.

Altogether, our previous [18] and present results indicate that GPC3 reexpression regulates Wnt, PKB/Akt and p38MAPK signaling pathways in mammary tumor cells. The alterations of these pathways could be responsible for the impairment of the in vivo metastatic capacity detected in LM3-GPC3 cells. Added to the GPC3 induced reduction in LM3 cells clonogenic ability previously reported [12], GPC3 inhibitory effect on mammary tumor metastasis could be explained by an increase in the susceptibility to cell death mainly mediated by p38MAPK and PKB/Akt signaling modulation.

We believe that the understanding of these mechanisms will help us to understand how GPC3 disables in vivo metastasis, being this of potential application in breast cancer therapy.

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References

1. Filmus J, Selleck SB (2001) Glypicans: proteoglycans with a surprise. J Clin Invest 108:497–501
2. Nakato H, Futch TA, Selleck SB (1995) The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division pattering during postembryonic development of the nervous system in Drosophila. Development 121:3687–3702
3. Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N (2001) Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development 126:87–94
4. Cano-Gauci DF, Song HH, Yang H, McKerlie C, Choo B, Shi W, Pullano R, Piscione TD, Grisaru S, Soon S, Sedlackova L, Tanswell AK, Mak TW, Yeger H, Lockwood GA, Rosenblum ND, Filmus J (1999) Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J Cell Biol 146:255–264
5. Chiao E, Fisher P, Crisponi L, Deiana M, Dragatsis I, Schlessinger D, Pilia G, Efstratiadis A (2002) Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling. Dev Biol 243:185–206. doi:10.1006/dbio.2001.0554
6. Garganta CL, Bodurtha JN (1992) Report of another family with Simpson-Golabi-Behmel syndrome and a review of the literature. Am J Med Genet 44:129–135. doi:10.1002/ajmg.1320440202
7. Pilia G, Hughes-Benzie RM, MacKenzie A, Baybayan P, Chen EY, Huber R, Neri G, Cao A, Forabosco A, Schlessinger D (1996) Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet 12:241–247. doi:10.1038/ng0396-241
8. Dueñas-Gonzalez A, Kaya M, Shi W, Song H, Test JR, Penn LZ, Filmus J (1998) OCI 5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell-specific manner. J Cell Biol 141:1407–1414. doi:10.1083/jcb.141.6.1407
9. Lin H, Huber R, Schlessinger D, Morin PJ (1999) Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res 59:807–810
10. Murthy SS, Shen T, De Rienzo A, Lee WC, Ferriola PC, Jhanwar SC, Mossman BT, Filmus J, Testa JR (2000) Expression of GPC3, an X-linked recessive overgrowth gene, is silenced in malignant mesothelioma. Oncogene 19:410–416. doi:10.1038/sj.onc.1203322
11. Xiang YY, Ladeda V, Filmus J (2001) Glypican-3 expression is silenced in human breast cancer. Oncogene 20:7408–7412. doi:10.1038/sj.onc.1204925
12. Peters MG, Farias E, Colombo L, Filmus J, Puricelli L, Bal de Kier Joffe E (2003) Inhibition of invasion and metastasis by glypican-3 in a syngeneic breast cancer model. Breast Cancer Res Treat 80:221–232. doi:10.1023/A:1024549729256
13. Weksberg R, Squire JA, Templeton DM (1996) Glypicans: a growing trend. Nat Genet 12:225–227. doi:10.1038/ng0396-225
14. Song HH, Shi W, Filmus J (1997) OCI-5/rat glypican-3 binds to fibroblast growth factor-2 but not to insulin-like growth factor-2. J Biol Chem 272:7574–7577. doi:10.1074/jbc.272.12.7574
15. Chiao E, Fisher P, Crisponi L, Deiana M, Dragatsis I, Schlessinger D, Pilia G, Efstratiadis A (2002) Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling. Dev Biol 243:185–206. doi:10.1006/dbio.2001.0554
16. Capurro MI, Xiang YY, Lobe C, Filmus J (2005) Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res 65:6245–6254. doi:10.1158/0008-5472.CAN-04-4244
17. Song HH, Shi W, Xiang YY, Filmus J (2005) The loss of glypican-3 induces alterations in Wnt signaling. J Biol Chem 280:2116–2125. doi:10.1074/jbc.M410090200
18. Stigliano I, Puricelli L, Filmus J, Sogayar MC, Bal de Kier Joffe E, Peters MG (2008) Glypican-3 regulates migration, adhesion and actin cytoskeleton organization in mammary tumor cells through Wnt signaling modulation. Breast Cancer Res Treat
19. Urtreger A, Ladeda V, Puricelli L, Rivelli A, Vidal MC, Lustig ES, Bal de Kier Joffé E (1997) Modulation of fibronectin expression and proteolytic activity associated with the invasive and metastatic phenotype in two murine mammary cell lines. Int J Oncol 11:489–496
20. Bal de Kier Joffé E, Puricelli L, Vidal MC, Lustig ES (1983) Characterization of two murine mammary adenocarcinoma tumors with different metastatic ability. J Exp Clin Cancer Res 2:151–160
21. Mizushima S, Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res 18:5322. doi:10.1093/nar/18.17.5322
22. Filmus J, Shi W, Wong ZM, Wong MJ (1995) Identification of a new membrane-bound heparan sulphate proteoglycan. Biochem J 311(Pt 2):561–565
23. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3
24. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. doi:10.1093/nar/29.9.e45
25. Mitsiades CS, Mitsiades N, Koutsilieris M (2004) The Akt pathway: molecular targets for anti-cancer drug development. Curr Cancer Drug Targets 4:235–256. doi:10.2174/1568009043333032
26. Gonzalez AD, Kaya M, Shi W, Song H, Testa JR, Penn LZ, Filmus J (1998) OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol 141:1407–1414. doi:10.1083/jcb.141.6.1407
27. Berger JC, Vander Griend D, Stadler WM, Rinker-Schaeffer C (2004) Metastasis suppressor genes: signal transduction, cross-talk and the potential for modulating the behavior of metastatic cells. Anticancer Drugs 15:559–568. doi:10.1097/01.cad.0000132233.36512.fa
28. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70. doi:10.1016/S0092-8674(00)81683-9
29. Pilia G, Hughes-Benzie RM, MacKenzie A, Baybayan P, Chen EY, Huber R, Neri G, Cao A, Forabosco A, Schlessinger D (1996) Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet 12:241–247. doi:10.1038/ng0396-241
30. Nakano K, Vousden KH (2001) PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7:683–694. doi:10.1016/S1097-2765(01)00214-3
31. Vousden KH, Lu X (2002) Live or let die: the cell's response to p53. Nat Rev Cancer 2:594–604. doi:10.1038/nrc864
32. Platanias LC (2003) Map kinase signaling pathways and hematologic malignancies. Blood 101:4667–4679. doi:10.1182/blood-2002-12-3647
33. Baldwin AS Jr (2001) Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest 107:3–6. doi:10.1172/JCI11891
34. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A (2005) Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell 20:539–550. doi:10.1016/j.molcel.2005.10.033

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MS Code : BREA3611 | CP | DISK

35. Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911–1912. doi:10.1126/science.1072682
36. Huang J, Wu L, Tashiro S, Onodera S, Ikejima T (2005) Bcl-2 up-regulation and P-p53 down-regulation account for the low sensitivity of murine L929 fibrosarcoma cells to oridonin-induced apoptosis. Biol Pharm Bull 28:2068–2074. doi:10.1248/bpb.28.2068
37. She QB, Chen N, Dong Z (2000) ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. J Biol Chem 275:20444–20449. doi:10.1074/jbc.M001020200
38. Baeg GH, Perrimon N (2000) Functional binding of secreted molecules to heparan sulfate proteoglycans in Drosophila. Curr Opin Cell Biol 12:575–580. doi:10.1016/S0955-0674(00)00134-4
39. Katoh M (2005) WNT/PCP signaling pathway and human cancer. Oncol Rep 14:1583–1588 (review)
40. Kuriyama M, Harada N, Kuroda S, Yamamoto T, Nakafuku M, Iwamatsu A, Yamamoto D, Prasad R, Croce C, Canaani E, Kaibuchi K (1996) Identification of AF-6 and canoe as putative targets for Ras. J Biol Chem 271:607–610. doi:10.1074/jbc.271.2.607
41. Cuadrado A, Lafarga V, Cheung PC, Dolado I, Llanos S, Cohen P, Nebreda AR (2007) A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. EMBO J 26:2115–2126. doi:10.1038/sj.emboj.7601657

Journal : Large 10549 | Dispatch : 7-3-2009 | Pages : 16
Article No. : 362 | ☐ LE | ☐ TYPESET
MS Code : BREA3611 | ☑ CP | ☑ DISK