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Growth hormone-releasing hormone (GHRH) promotes metastatic phenotypes through EGFR/HER2 transactivation in prostate cancer cells

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Abstract

The involvement of growth hormone-releasing hormone (GHRH) in several relevant processes that contribute to prostate cancer progression was analyzed. Firstly, we evaluated GHRH effects on cell proliferation and adhesion in human cancer prostate cell lines, LNCaP and PC3, by using specific assays (BrdU incorporation and collagen adhesion). The expression levels of the main marker molecules of these processes were measured by RT-PCR, Western blotting and zymography assays. GHRH increased both cell proliferation and proliferating cell nuclear antigen (PCNA) levels in LNCaP cells and in PC3 cells; however, such a rise was faster in the PC3 cells that represent the most aggressive stage of prostate cancer. Furthermore, GHRH significantly reduced cell adhesion and E-cadherin levels in LNCaP and PC3 cells and up-regulated the total and nuclear expression of β-catenin in PC3 cells. In addition, we assessed cell cycle, cell migration and VEGF secretion in PC3 cells. GHRH augmented the number of cells in G2/M-phase but diminished that corresponding to G1-phase. Cell-cycle specific markers were evaluated since GHRH effects may be related to their differential expression; we observed a decrease of p53, p21, and Bax/Bcl2 ratio. Furthermore, GHRH increased the expression of CD44, c-myc and cyclin D1, MMP-2 and MMP-9 activity, and VEGF secretion. We also observed that EGFR and/or HER2 transactivation is involved in cell adhesion, cell migration and VEGF secretion produced by GHRH. Consequently, present results define GHRH as a proliferative, anti-apoptotic and migratory agent in prostate cancer.

Keywords: GHRH, Cell adhesion, Cell migration, VEGF secretion, Prostate cancer

1. Introduction

Prostate cancer (PC) is the second most common cause of cancer death in men worldwide (Siegel et al., 2016). Progression of prostate cancer results in metastases in bone and other tissues, causing bone pain, skeletal complications, and patient mortality. Thus, the knowledge of molecular events leading to prostate cancer progression is essential for the development of improved therapies for patients with advanced prostate cancer.

Growth hormone-releasing hormone (GHRH) is a neuropeptide hormone, secreted by the hypothalamus, which binds to its receptor (GHRH-R) in pituitary somatotrophs and activates synthesis and secretion of growth hormone (GH) (Barabutis and Schally, 2010; Kiaris et al., 2011). Both, GHRH and its receptors (pituitary type and truncated splice variants), are expressed by various extrahypothalamic sites as observed in surgical specimens and tumor cell lines of a diversity of human cancers (Busto et al., 2002; Garcia- Fernandez et al., 2003; Halmos et al., 2002; Havt et al., 2005; Kahan et al., 1999). In fact, the presence of GHRH and SV1 isoform of GHRH receptors in LNCaP and PC3 cells have been previously described (Chopin and Herington, 2001). Moreover, there are various reports that support that GHRH and its tumoral SV receptors may form an autocrine mitogenic loop in prostate cancer LNCaP cell line (Barabutis and Schally, 2008a, 2008b; Plonowski et al., 2002). Furthermore, several *in vitro* and *in vivo* studies show the effect of antagonistic analogs of GHRH on cell proliferation and apoptosis in prostate cancer (Barabutis and Schally, 2008a, 2008b; Barabutis et al., 2010; Stangelberger et al., 2012; Muñoz-Moreno et al., 2013; Fahrenholtz et al., 2014). However, the effects of GHRH on processes that characterize a more aggressive molecular phenotype of prostate cancer have not been extensively studied.

On the other hand, it is known that G-protein coupled receptors (GPCRs) are able to activate tyrosine kinase receptors (RTKs) (Delcourt et al., 2007; Pyne and Pyne, 2011).

Interestingly, GHRH is involved in the transactivation of the signaling of epidermal growth factor receptor (EGFR/HER1/ErbB1) and human epidermal growth factor receptor-2 (HER2) in prostate cancer (Munoz-Moreno et al., 2014). This fact is extremely important since the formation of EGFR/HER2 heterodimers is related to mitogenic signaling path- ways implicated in prostate cancer progression towards androgen independence (Di Lorenzo et al., 2002; Berger et al., 2006).

The beginning of the cancer process is related to both an un- controlled growth and loss of tumor cell adhesion. In regard to the former, numerous molecules regulate the cell cycle including the proliferating cell nuclear antigen (PCNA) that acts as a processivity factor for DNA polymerase d during DNA replication (Maga and Hubscher, 2003; Wang, 2014). In addition, p21 protein, a cyclin- dependent kinase (CDK) inhibitor, is capable to bind to both cyclin-CDK and PCNA. Through its binding to PCNA, p21 inhibits replication by blocking activity of PCNA to stimulate polymerases (Moldovan et al., 2007; Waga and Stillman, 1998). Furthermore, p21 is able to inhibit the binding to cyclin-CDK complex leading to cell growth arrest in cell cycle (Abbas and Dutta, 2009). The anti- proliferative actions of p21 may occur by a p53-dependent mechanism (Perez-Sayans et al., 2013). In addition, p53 induces apoptosis through the regulation of apoptotic genes. Thus, p53 activates and represses the transcription of Bax (pro-apoptotic) and Bcl2 (antieapoptotic), respectively, resulting in the activation of the programmed cell-death process (Mirzayans et al., 2012). Instead, the disruption of both cell-cell and cell-extracellular matrix interactions are mediated by cell adhesion molecules. Loss of one of them, E-cadherin, a member of the cadherin family, has been associated with the epithelial-mesenchymal transition (EMT), the mesenchymal-epithelial transition (MET) and a higher motility, invasiveness and resistance to apoptosis allowing aggressive phenotype in various human cancers (Margineanu et al.,

2008; Beuran et al., 2015; Le Bras et al., 2012; Onder et al., 2008). Furthermore, the cytoplasmic domain of E-cadherin recruits several molecules including β-catenin, providing anchorage to the cytoskeleton (Schmalhofer et al., 2009). The β-catenin also acts as a transcriptional co-activator in the Wnt signaling pathway used during development to control cell fate decisions and implicated in many cancers (Schmalhofer et al., 2009; Howard et al., 2011; Huber and Weis, 2001). Loss of E-cadherin causes β-catenin release from the membrane, which is associated with the transcription factor Lef/TCF (Lymphoid enhancer factor/T cell factor) in the cytoplasm. This complex translocates to the nucleus where β-catenin-Lef/TCF activates transcription of genes involved in tumor progression such as cyclin D1, CD44, cemyc, metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) (Thakur and Mishra, 2013). Furthermore, metastatic process that starts with the disruption of cell-cell local interaction and alteration of basement membrane carry on with invasion and infiltration of surrounding tissue, and penetration into blood or lymphatic vessels with consequent transportation of neoplastic cells through the blood circulation (Hanahan and Weinberg, 2011).

The aim of this study was to determine the role of GHRH in the progression of prostate cancer by analyzing the participation of this hormone in important cellular processes such as cell proliferation, cell adhesion, cell migration and angiogenesis, typically associated with metastatic phenotypes in prostate cancer. In addition, we observed the involvement of EGFR and/or HER2 in the effects of GHRH on such processes.

2. Materials and methods

2.1. Peptides

GHRH (1-29)NH_2 was purchased from PolyPeptide (Strasbourg, France), EGFR tyrosine kinase inhibitor (AG-1478) from Calbiochem (Darmstadt, Germany), and HER-2 tyrosine kinase inhibitor (AG- 825) from Tocris Bioscience (Bristol, United Kingdom).

2.2. Cell cultures

Two human prostate cancer cell lines that exhibit different features of prostate cancer progression were used. LNCaP is an androgen-responsive cancer cell line which may represent early stage of the disease. PC3 is an androgen-unresponsive cell line that may represent recurrent prostate cancers that have achieved androgen independence. Cell lines were obtained from the American Type Culture Collection. LNCaP and PC3 cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). All culture media were also supplemented with 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B from Life Technologies, Carlsbad, CA, USA). The culture was performed in a humidified 5% CO_2 environment at 37 ^{o}C. After the cells reached 70-80% confluence, they were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin/0.2% EDTA, and seeded at 30,000-40,000 cells/cm^2. The culture medium was changed every 3 days.

2.3. Cell proliferation assays

LNCaP and PC-3 cells (2 x 10^5 cells) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B) for 16 h. Then, cells were subjected for 24 h to different treatments. In the last 30 min of incubation, cells

were labeled with 10 μM bromodeoxyuridine (BrdU). Thereafter, cells were washed with PBS, fixed with ice-cold absolute ethanol, and stored at -20 ^{o} C for 30 min. Fixative was removed by centrifugation and pellets were washed with PBS. DNA was partially denatured by incubation with 1 N HCl for 30 min at room temperature and then cells were washed three times with PBS containing 0.05% Tween-20 (pH 7.4) and 0.1% BSA. Cells were incubated with 20 μl of anti-BrdU monoclonal antibody with FITC (BD Bioscience, San Agustin de Guadalix, Spain) for a 30 min-period in darkness. For flow cytometry analysis, cells were stained with propidium iodide (PI) staining solution (50 μg/ml PI and 10 μg/ml RNase). The number of BrdU-positive cells was counted using a FACSCalibur cytometer (BD Bioscience). Results obtained were analyzed with the Cyflogic v 1.2.1 program.

2.4. Cell adhesion assays

Concentrated type-I collagen solution was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 h at 37 ^{o}C. Plates were washed twice with PBS (pH 7.4). Cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were resuspended in RPMI medium/0.1% (w/v) BSA (pH 7.4) and treated with 0.1 μM GHRH for 30 min. Then, LNCaP and PC3 cells were plated at 2.5 x 10^4 cells per 100 μl. The assay was terminated at the indicated time intervals by aspiration of the wells. Cell adhesion was quantified by adding 1 mg/ml of the substrate 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) followed by 1 h incubation. Isopropanol (50 μl) was added to each well to dissolve the dark blue formazan precipitates and absorbance was read at 570 nm in a plate reader (ELX 800, Bio-Tek Instruments, Winooski, VT, USA). Results were expressed as the relative percentage of absorbance compared with the corresponding control cells.

2.5. Isolation of cell lysates

LNCaP and PC3 cells (1.5-3 x 10^6 cells) were washed with ice-cold PBS and then harvested, scraped into ice-cold PBS, and pelleted by centrifugation at 500x*g* for 5 min at 4 °C. For preparation of cell lysates, cells were kept on ice for 30 min in a solution containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. Thereafter, cells were pelleted by centrifugation at 4000x*g* for 5 min at 4 °C.

2.6. Western blot assays

Proteins from cell lysates extracts (30 μg) were denatured by heating. Then, they were resolved by 10% SDS-PAGE, and blotted onto a nitrocellulose membrane (BioTrace/NT) overnight in 50 mM Tris-HCl, 380 mM glycine, 0.1% SDS, and 20% methanol. Anti-PCNA (Zymed), anti-E-cadherin and anti-β-catenin (BD Bioscience), anti-p53 (Sigma-Aldrich), anti-Bcl2 and anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were added followed by incubation for 1 h at room temperature. After treatment for 1 h at room temperature with the corresponding secondary antiserum (anti-rabbit or anti-mouse sera), the signals were detected with enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA) using β-actin antibody as loading control.

2.7. Cell cycle assays

PC3 cells (2 x 10^5) were grown in 6-well plates. After 24 h, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B) for 16 h. After that, cells were subjected to various treatments for 8 h. Then, the cells were washed with PBS and

detached with 0.25% trypsin/0.2% EDTA. After centrifugation at 500x*g* for 5 min at 4 ^{o}C, the pellets were mixed with ice-cold 70% ethanol and then kept at -20 ^{o}C for 30 min. After ethanol removing by centrifugation, the pellets were washed with PBS and centrifuged again. The supernatants were discarded and the pellets suspended in PBS, 0.2 mg/ml RNase A and 20 μg/ml PI before flow cytometry analysis. Results obtained were analyzed with the Cyflogic v 1.2.1 program.

2.8. RNA isolation and RT-PCR

PC3 cells were placed in 6-well plates (15 x 10^3 cells) and incubated with various agents in serum medium for different periods. Total RNA was isolated with Tri-Reagent (Sigma) according to the instructions of the manufacturer. Two micrograms of total RNA were reverse-transcribed using 6 μg of hexamer random primer and 200 U M-MLV RT (Life Technologies) in the buffer supplied with the enzyme, supplemented with 1.6 μg/ml oligo dT, 10 nM dithiothreitol (DTT), 40 U RNasin (Promega Madison, WI, USA), and 0.5 mM deoxyribonucleotides (dNTPs). Two microliters of the RT reaction were used for PCR amplification with a primer set which amplifies cDNAs for p53, p21, CD44, cyclin D1, c-myc, MMP9 y MMP2 or β-actin. The corresponding sequences of oligonucleotide primers appear in Table 1. PCR-conditions were: denaturation at 94 ^{o}C for 5 min, followed by 26-40 cycles of 95 ^{o}C for 1 min, 57 ^{o}C for 1 min, 72 ^{o}C for 1 min, and then a final cycle of 10 min at 72 ^{o}C. The signals were normalized with the β-actin gene expression level. The PCR products were separated by electrophoresis and visualized under UV light in 2% agarose gels, stained with GelRedTM nucleic acid gel stain (Biotium, Hayward, CA, USA).

2.9. Wound-healing assay

PC3 cells were incubated in 24-well plates and a small wound area was made in the

confluent monolayer with a scraper. Afterwards, cells were incubated in the absence or presence of GHRH (0.1 μM). Four representative fields of each wound were captured using a Nikon Diaphot 300 inverted microscopy at different times (0, 4, 8 and 24 h). Wound areas of untreated samples were averaged and assigned a value of 100%.

2.10. Gelatin zymography

Zimography assays were carried out as described previously (Mun oz-Moreno et al., 2013). Briefly, the samples (6 μg of protein) were subjected to 10% SDS-PAGE with 0.1% (w/v) gelatin (Sigma) as the substrate. After staining, the activity of MMP2 and MMP9 as well as their corresponding proenzymes were semiquantitatively determined by densitometry.

2.11. Determination of VEGF levels

$VEGF_{165}$ levels were determined in tumor homogenates (15 μg) by using the human VEGF DuoSet (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Data were normalized by the protein concentration in each sample.

2.12. Data analysis

Quantification of band densities was performed using Quantitative One Program (Bio-Rad, Alcobendas, Spain). Data were subjected to one-way ANOVA and differences were determined by Bonferroni's multiple comparison test. Each experiment was repeated at least three times. Data are shown as the means of in- dividual experiments and presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of GHRH on cell proliferation in LNCaP and PC3 cells

Firstly, we performed viability studies using different concentrations of GHRH (10^{-9}-10^{-5}

M). GHRH significantly increased ($P < 0.01$) cell viability since 0.1 μM (data not shown). The effects of GHRH (0.1 μM) on proliferation were assessed by BrdU incorporation assays in LNCaP and PC3 cells (Fig. 1A). After 24 h of GHRH treatment, cell proliferation significantly increased in both cell lines. Thus, a higher cell proliferation occurred after GHRH- treatment in PC3 cells (51%, $P < 0.001$) as compared to than that in LNCaP cells (27%, $P < 0.01$).

Changes induced by GHRH in cell proliferation may be due to variations on the expression of specific markers such as PCNA. In this context, the hormone significantly increased the expression of PCNA in a time-dependent manner from 1 to 16 h, with a maximum value of expression at 8 h (90%, $P < 0.001$) in LNCaP cells (Fig. 1B). Similarly, GHRH provoked a substantial rise on PCNA protein levels from 1 to 8 h in PC3 cells; however, the maximum effect was recorded at 4 h (95%, $P < 0.001$).

3.2. Effect of GHRH on cell cycle in PC3 cells

In order to know whether the changes in proliferation of PC3 cells is correlated to variations in cell cycle, analysis of such a process by flow cytometry was carried out. Results revealed that treatment with GHRH (0.1 μM) produced major changes in the distribution of the cells across the different phases of cell cycle (Fig. 2A). In the control group, 54.42 ± 0.67% of the cells were in G0/ G1 phase, 11.78 ± 0.14% in S phase, and 25.52 ± 0.73% in G2/M phase. After 8 h-treatment with GHRH, the distribution was the following: 39.30 ± 0.75%, 9.50 ± 0.91%, and 32.49 ± 1.01% for G0/G1, S and G2/M phases, respectively. GHRH significantly inhibited ($P < 0.001$) the proportion of cells in the G1 phase.

With the purpose to investigate whether GHRH effects on the cell cycle were related to changes in proteins associated to the control of growth arrest and apoptosis, we analyzed

the expression of p53, p21, Bax, and Bcl2 in PC3 cells. The results show a significant reduction of p53 mRNA levels (by 15-16%, $P < 0.01$) after 30-60 min of GHRH-treatment (Fig. 2B), which correlated with a substantial decrease in protein expression (around 30-42%, $P < 0.001$) from 30 to 60 min (Fig. 2C). In addition, p21 mRNA levels decreased slightly (by 30%, $P < 0.01$) after 2 h of treatment with GHRH (Fig. 2D). Regarding to apoptotic molecules, GHRH decreased Bax and increased Bcl2 protein levels (Fig. 2E). Therefore, the Bax/ Bcl-2 ratio (Fig. 2E) indicated a significant decrease in a time- dependent manner, from 2 to 24 h, with a minimum value at 16 h (around 50%, $P < 0.001$) which confirms a reduced programmed cell death.

3.3. Effect of GHRH on cell adhesion in LNCaP and PC3 cells

GHRH effect on cell adhesion was assessed by means of assays of collagen adhesion in LNCaP and PC3 cells. Cells were suspended and incubated for 30 min with 0.1 μM GHRH. Then, cells placed in 96-well plates with collagen were removed at different times (Fig. 3A). Thereafter, cells were incubated with MTT and assessed by cell adhesion assay. Results showed that treatment with GHRH outcomes in decreased adhesion in the two cell lines studied. GHRH-treatment significantly reduced ($P < 0.01$) by 22% cell adhesion after 80 min in LNCaP cells. In PC3 cells, the hormone significantly diminished (P < 0.001) cell adhesion by 42% from 10 to 80 min.

In order to know whether changes of cell adhesion were related to modification of the levels of adhesion markers, the expression of E-cadherin and β-catenin was assessed after cell treatment with GHRH (0.1 μM) at different periods. The results shown in Fig. 3B indicate that GHRH significantly decreased ($P < 0.001$) E-cadherin protein levels in both tumor cell lines (58% in LNCaP and 80% in PC3 cells) after 8 h, compared with control values.

In order to know more about the formation of the complex E-cadherin-β-catenin, we evaluated the expression of β-catenin by Western blotting with a specific antibody after GHRH-treatment of cells at different times. Protein levels of β-catenin increased in both tumor cell lines after 1 h of treatment with the hormone. Results show that GHRH significantly increased the expression of β-catenin in a time-dependent manner from 1 to 8 h, with a maximum level of expression at 8 h (80%, $P < 0.001$) in LNCaP cells (Fig. 3C). Similarly, GHRH provoked a substantial rise of β-catenin protein levels from 0.5 to 24 h (79%, $P < 0.001$). Interestingly, the effects of GHRH on β-catenin correlated with those observed for E-cadherin.

3.4. Effect of GHRH on the cell distribution of β*-catenin in PC3 cells*

The subcellular distribution of β-catenin is a key element to assure its specific function in each cell compartment. For this reason, β-catenin protein expression was analyzed in cytosol and nucleus after cell treatment with GHRH (0.1 μM). PC3 cell line was chosen, since a further increase in the expression of β-catenin occurred after exposition to the hormone. Results show that levels of β-catenin significantly decreased ($P < 0.01$) by 70% in cytosol after 2 h of GHRH treatment. In nuclei, the rise of protein expression became significant ($P < 0.01$) at 1 h of GHRH-treatment and was maintained until 24 h (35-57%) (Fig. 4A).

The increase of β-catenin protein levels in the nucleus of PC3 cells could result in induction of the transcription of important target genes such as CD44, cyclin D1 and c-myc. Therefore, mRNA levels corresponding to these genes were analyzed by RT-PCR after treatment of PC3 cells with 0.1 μM GHRH (Fig. 4B). A time-dependent increase of mRNA levels was observed, with the exception of c-myc at 6 h. Maximum values of mRNA levels were reported at 1 h, 6 h, and 2 h for CD44 (40%, $P < 0.001$), c-myc (45%, $P < 0.01$), and

cyclin D1 (50%, $P < 0.001$), respectively.

3.5. Effect of GHRH on cell migration in PC3 cells

Tumor cells begin to migrate to other tissues after the loss of cell adhesion. We evaluated the migratory capability of PC3 cells after treatment with GHRH by wound-healing assays. PC3 cell line was selected because of a relevant effect on the adhesion after GHRH-treatment. The results (Fig. 5A) show that cell treatment with GHRH caused a faster closure of the wound as compared to un-treated PC3 cells. Total closure occurred after 24 h of exposition to the hormone while almost 25% ($P < 0.001$) of the wound remained unsealed in untreated cells.

MMP2 and MMP9 metalloproteinases are directly involved in cell migratory processes. Thus, we assessed such enzymes at two levels: mRNA expression levels and gelatinolytic activity at different times after GHRH treatment (Fig. 5B). Levels of mRNA for MMP9 increased from 45 min (33%, $P < 0.01$) up to 2 h of exposition to the hormone. MMP2 mRNA levels increased from 15 min (39%, $P < 0.01$) up to 1 h after GHRH treatment. Importantly, augmented expression of mRNA levels resulted in increased gelatinolytic activities of both enzymes (Fig. 5C). The relative presence of pro-MMP9 and pro-MMP2 significantly increased (by 50%, $P < 0.01$, and 91%, $P < 0.001$, respectively), at 2 h of treatment with GHRH.

3.6 Effect of GHRH on $VEGF_{165}$ secretion in PC3 cells

VEGF is the major pro-angiogenic factor involved in tumor progression. It is directly related to molecules involved in migration and adhesion such as β-catenin and MMP9. Therefore, we assessed by ELISA the $VEGF_{125}$ levels released by the cells to the extracellular medium after treatment with GHRH at different times. The hormone elicited a significant peak (52%, $P < 0.001$) of VEGF secreted by the cells after 24 h of cell exposition (Fig. 5D).

The content of extracellular VEGF at time 0 was 355 ± 62 pg/ml.

3.7. Involvement of GHRH-induced transactivation of EGFR or HER2 on cell adhesion, cell migration and VEGF secretion in PC3 cells

Previous studies demonstrate that GHRH stimulates a rapid ligand-independent activation of EGFR and HER2 involving at least cAMP/PKA and Src family signaling pathways. Moreover, GHRH also stimulates a slow ligand-dependent activation of EGFR and HER2 involving an extracellular pathway with an important role for ADAM (Munoz-Moreno et al., 2014). Therefore, we assessed whether the effects of GHRH on the processes of cell adhesion, migration and VEGF secretion were mediated by EGFR/HER2 transactivation in PC3 cells. For this purpose, specific inhibitors of the tyrosine kinase activity for EGFR (AG-1478) and/or for HER2 (AG-825) were used in the assays. Cells were pre-treated with each of the inhibitors (10 μM) for 30 min, and then incubated with GHRH (0.1 μM) at the same times in which the transactivation occurs (30 s and 30 min) (Munoz-Moreno et al., 2014).

Results from cell adhesion assays are shown in Fig. 6A. GHRH alone provoked a reduction in cell adhesion at two times studied (42% at 30 s, $P < 0.01$-59% at 30 min, $P < 0.001$). However, when PC3 cells were exposed to specific inhibitors of HER2 (AG-825) or EGFR (AG-1478), GHRH was unable to decrease levels of cell adhesion at any of the times studied (14-35%). This fact could indicate that both EGFR and HER2 are involved in the mechanism of action of GHRH on cell adhesion process in PC3 cells.

VEGF secretion could be regulated by EGFR since the use of the specific EGFR inhibitor blocked the stimulatory effect of GHRH treatment on this process at 30 min (28%) (Fig. 6B). However, phosphorylation inhibitor of HER2 did not modify the effects of GHRH. Cell migration seems to be mediated by activation of EGFR because the use of the AG-

1478 provoked the decrease the capability to mobility of PC3 cells after 24 h of treatment with GHRH (Fig. 6C). Conversely, AG-825 did not modify the effects of GHRH. Therefore, EGFR transactivation, but not HER2, could be involved in the cell migration and secretion of VEGF produced by GHRH.

4. Discussion

The presence of GHRH and its receptors has been described in prostate cancer specimens and androgen-dependent prostate cancer cell lines (Halmos et al., 2002; Chopin and Herington, 2001; Barabutis and Schally, 2008b). In addition, GHRH and its tumoral SV receptors may form an autocrine mitogenic loop in an androgen-dependent prostate cancer LNCaP cell line (Barabutis and Schally, 2008a, 2008b; Plonowski et al., 2002). In the present work, GHRH modulated the proliferation of prostate cancer cells, causing a greater response in the cell line related to advanced prostate cancer. Changes in cell proliferation induced by GHRH may be the result of differential expression of a major proliferation marker, PCNA (Stoimenov and Helleday, 2009; Barabutis et al., 2010). In this regard, expression levels of PCNA were elevated in androgen- dependent cancer LNCaP cells exposed to GHRH and decreased in cells cultured with GHRH antagonist (Barabutis and Schally, 2008b). In our study, after treatment of both cell lines with GHRH, PCNA levels increased showing a rapid and greater response in the more aggressive tumor cells.

The effect of GHRH on cell cycle correlates with findings from cell proliferation assays in advanced prostate cancer. Thus, GHRH increased the number of cells in mitosis explaining the rise on cell proliferation. Furthermore, the effect of GHRH on cell cycle could be due to changes on the expression of molecules such as p53, p21, Bax, and Bcl2. Interestingly, the ratio of proapoptotic/antiapoptotic (Bax/Bcl2) markers was reduced after treatment with 0.1 μM GHRH. This fact is crucial for preventing the initiation of an

apoptotic process and consequently maintain cell survive in cancer progression. Modifications in the content of such markers would define GHRH as an antiapoptotic factor in prostate cancer. In this regard, it has been described the effects of GHRH and GHRH antagonist on p53 protein expression in androgen-dependent prostate cancer LNCaP cells (Barabutis and Schally, 2008b). Additionally, *in vivo* studies show the inhibitory effect of antagonistic analogs of GHRH on experimental prostate cancers (Stangelberger et al., 2012; Muñoz-Moreno et al., 2013; Fahrenholtz et al., 2014).

In normal tissue, epithelial cells are interconnected by adhesive molecules that allow cells to maintain contact each other and with structure of extracellular matrix. However, tumor cells “disrupt” these bonds and “escape” to other parts of the body, resulting in metastasis. The first step to tumor progression is the loss of cell adhesion. In this regard, our results demonstrate the involvement of GHRH in the reduction of tumor cell adhesion being greater in advanced prostate cancer. E-cadherin is considered an anti- migration protein and a key marker of the epithelial phenotype. Binding of cells by E-cadherin inhibits cell motility, maintains normal epithelial phenotype and contribute to cell-cell adhesion (Arya et al., 2006; Farahani et al., 2014). In addition, changes in levels of E-cadherin have been associated with both EMT and MET processes in metastatic cancer (Onder et al., 2008; Heerboth et al., 2015). In this regard, it is important to note that the reduction of E- cadherin induced by GHRH could support the start of the EMT process. The β-catenin is a key component of the cadherin complex and plays a central role on Wnt pathway signaling (Thakur and Mishra, 2013). In epithelial cells, its association with E-cadherin prevents the release of β-catenin to the cytoplasm and its subsequent translocation to the nucleus where it can activate transcription of various genes (Orsulic et al., 1999; Su et al., 2015). Present results show that GHRH

increased the total and nuclear expression of β-catenin in tumor cells, with a higher response in advanced prostate cancer representative cells. It indicates that GHRH would activate Wnt pathway signaling through the nuclear translocation of β-catenin. On the other hand, the presence of GHRH increased β-catenin nuclear while reduced β-catenin cytosolic in androgen-unresponsive prostate cancer cells. Nuclear accumulation of β-catenin activates transcription of target genes, related to proliferation, migration, invasion and angiogenesis (Luu et al., 2004; Chiurillo, 2015). In our study, GHRH increased mRNA levels of c-myc, cyclin D1 and CD44, and protein levels of VEGF, MMP2 and MMP9 in PC3 cells through the nuclear translocation of β-catenin. In this regard, it has described that GHRH antagonist decreases expression of VEGF in human lung cancer cells (Siejka et al., 2012). Taken all together, these factors with the increased migratory capability could support the important role of GHRH promoting carcinogenic process.

In tumor cells, upregulating EGFR signaling is able to disrupt the cell adherence junctions and consequently, to induce cell migration and angiogenic process (Cheng et al., 2013; Gross et al., 2009). Furthermore, we have previously demonstrated EGFR/HER2 transactivation induced by GHRH and vasoactive intestinal peptide (VIP) in prostate cancer cells (Munoz-Moreno et al., 2014; Sotomayor et al., 2007). Current results indicate that both tyro- sine kinases receptors, EGFR and HER2, could be involved in the mechanism of action of GHRH on cell adhesion process in androgen-unresponsive prostate cancer cells. However, the secretion of VEGF and cell migration in PC3 cells seems to be mediated by EGFR but not by HER2. In this regard, it is known that EGFR/ HER2 activation leads to internalization of E-cadherin by the formation and activation of a signaling complex in which Src is a key component (Gross et al., 2009; Zhang et al., 2012). Moreover, the activation of EGFR on

tumor cells increase VEGF production and contribute to angiogenesis (Ciardiello et al., 2006). Thus, GHRH- EGFR crosstalk in the pathophysiology of prostate cancer provides greater opportunities for the identification of new therapeutic agents.

In summary, the current study provides a further elucidation of the mechanism of action of GHRH on processes as cell cycle, cell proliferation, cell adhesion, cell migration and angiogenesis of prostate cancer cells. Furthermore, EGFR/HER2 transactivation induced by GHRH is involved in cell adhesion, cell migration and angiogenesis process. Consequently, present results support to propose GHRH as a protumoral agent in prostate cancer.

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Declaration of interest

None of the authors has any conflict of interest regarding this study.

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Figure Legends

Fig. 1. Effect of GHRH (0.1 μM) on cell proliferation (A) and on PCNA expression (B) as assessed by BrdU incorporation and Western blot assays, respectively, in LNCaP and PC3 cells. Results are expressed as a percentage of control value. Data are mean ± SEM of ten independent experiments; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 2. Analysis of cell cycle (A), expression levels of p53 mRNA (B) and p53 protein (C), p21 mRNA (D), and apoptotic proteins (Bax and Bcl2) (E) after treatment with 0.1 μM of GHRH at different times in PC3 cells. The studies were performed by flow cytometry (A), RT-PCR (B and D) and Western blot (C and E) assays. Results from measurement of optical densities are expressed as a percentage of control value. Data from evaluation of apoptotic proteins are expressed as Bax/Bcl2 ratio. Data are mean ± SEM of four independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 3. Effect of GHRH (0.1 μM) on cell adhesion, as assessed by collagen adhesion (A), and expression of E-cadherin (B) and β-catenin (C), as measured by Western blotting, in LNCaP and PC3 cell lines. Results are represented as optical density units relative to untreated cells. Data are mean ± SEM of five independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 4. Effect of GHRH (0.1 μM) on the localization of β-catenin (A) and expression of mRNA for CD44, c-myc and cyclin D1 (B) in PC3 cells. The studies were performed by Western blot assays (A) and RT-PCR (B). Results are expressed as a percentage relative to its corresponding control value. Data are mean ± SEM of three independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 5. Analysis of cell migration (A), expression of mRNA (B) and protein (C) of MMP2 and MMP9, and $VEGF_{125}$ secretion after treatment with 0.1 μM GHRH at different periods in PC3 cells. The studies were performed using wound-healing (A), RT-PCR (B), zymography (C) and ELISA (D). Data are mean ± SEM of five independent experiments; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fig. 6. Involvement of EGFR and HER2 in the effect of GHRH on cell adhesion (A) (A), VEGF secretion (B) and cell migration (C) in PC3 cells. Cells were pre-treated for 30 min with 0.1 μM EGFR inhibitor, AG-1478 (10 μM), and HER2 inhibitor, AG-825 (10 μM) for 30 min, and then treated with 0.1 μM GHRH for 30 s or 30 min. The studies were performed using collagen cell adhesion (A), ELISA (B) and wound-healing (C) assays. Results are represented as optical density units relative to untreated cells. Data are mean ± SEM of three independent experiments; **, $P < 0.01$; ***, $P < 0.001$ versus the corresponding control; $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$ versus the corresponding GHRH treatment.

Table 1. Oligonucleotide primers used in RT-PCR analysis for p53, p21, CD44, cyclin D1, c-myc, MMP9, MMP2, and β-actin expression in human prostate PC3 cells. Primers were chosen with the assistance of the computer program Primer Express (Perkin Elmer Applied Biosystems).

mRNA	Primer	Sequence (5'→ 3')
p53	sense	AGGCCTTGGAACTCAAGG
	antisense	TGAGTCAGGCCTTCTGTCT
p21	sense	AGGTGAGGGGACTCCAAAGT
	antisense	ATGAAATTCACCCCCTTTCC
CD44	sense	AAGGTGGAGCAAACACAACC
	antisense	ACTGCAATGCAAACTGCAAG
cyiclin D1	sense	TTCGGGATGATTGGAATAGC
	antisense	TGTGAGCTGGTTCATTGAG
c-myc	sense	AGCGACTCTGAGGAGGAACA
	antisense	CTCTGACCTTTTGCCAGGAG
MMP9	sense	TGGGCTACGTGACCTATGACA
	antisense	TGTGGCAGCACCAGGGCAGC
MMP2	sense	ACCTGGATGCCGTCGTGGAC
	antisense	TGTGGCAGCACCAGGGCAGC
β-actin	sense	AGAAGGATTCCTATGTGGGCG
	antisense	CATGTCGTCCCAGTTGGTGAC

Figure 1

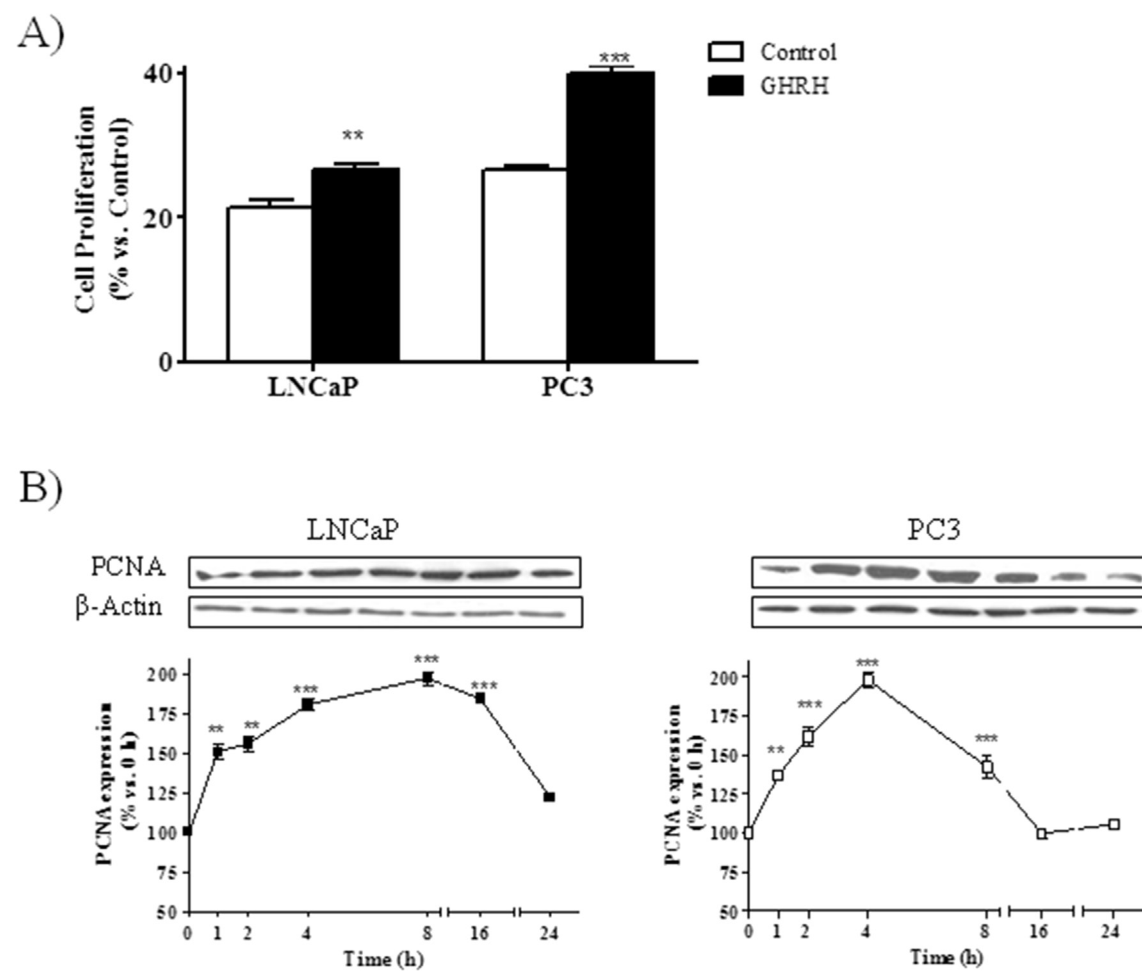
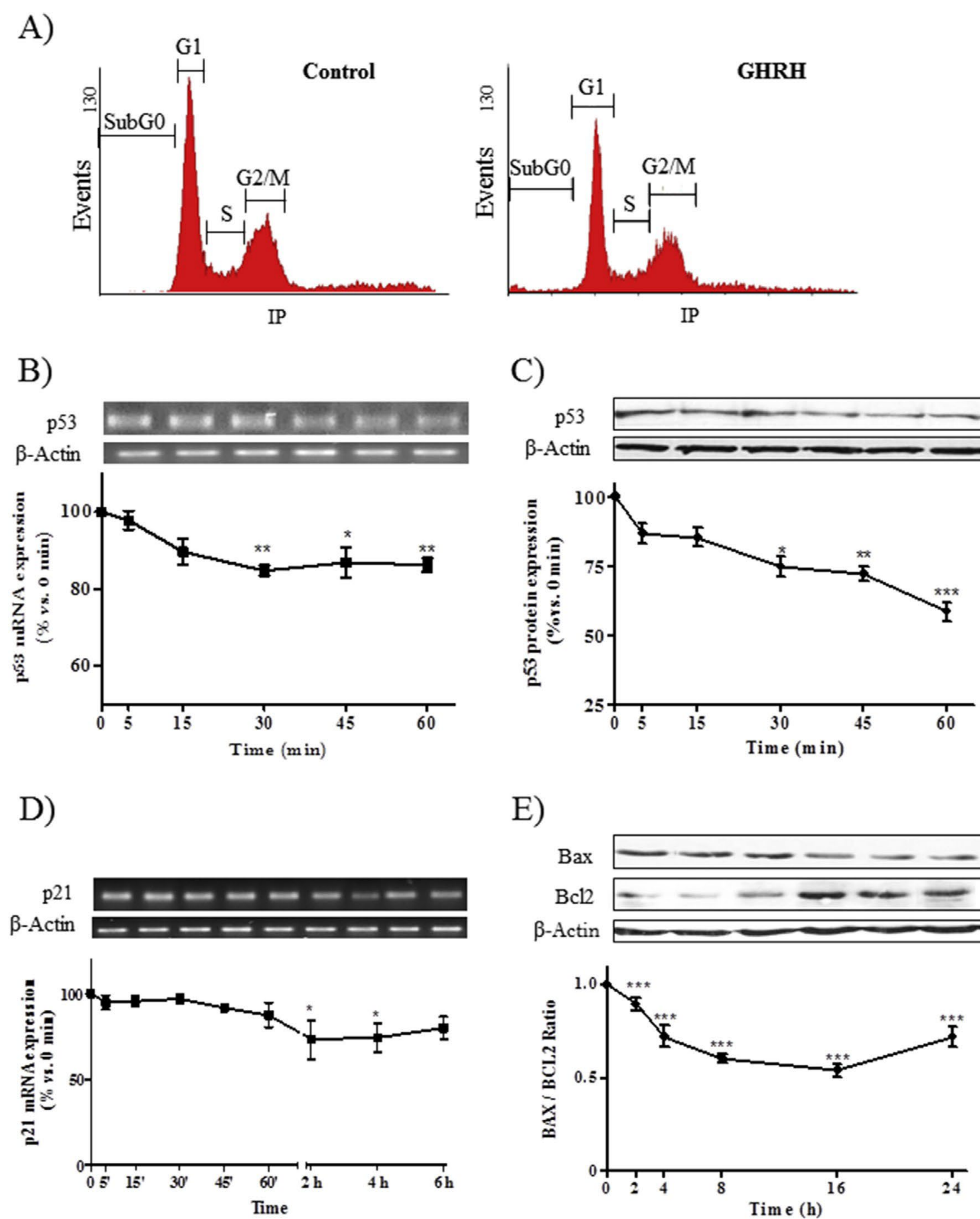


Figure 2



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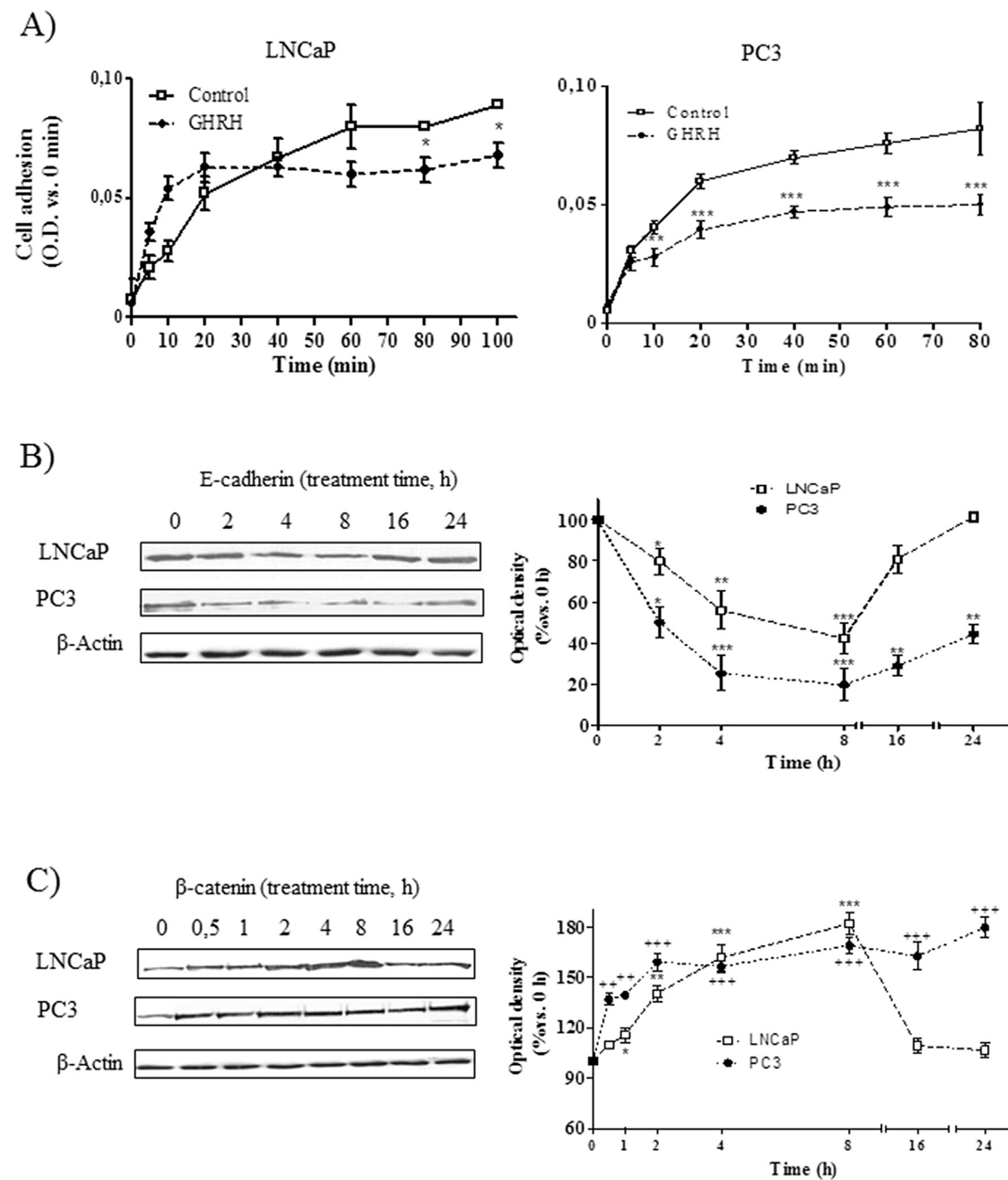
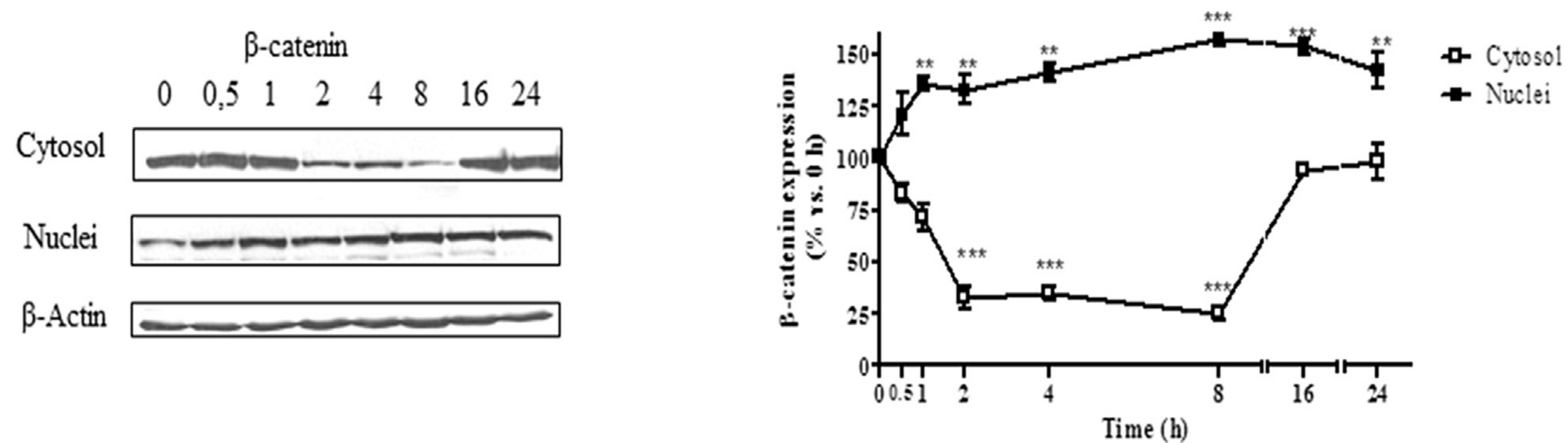


Figure 4



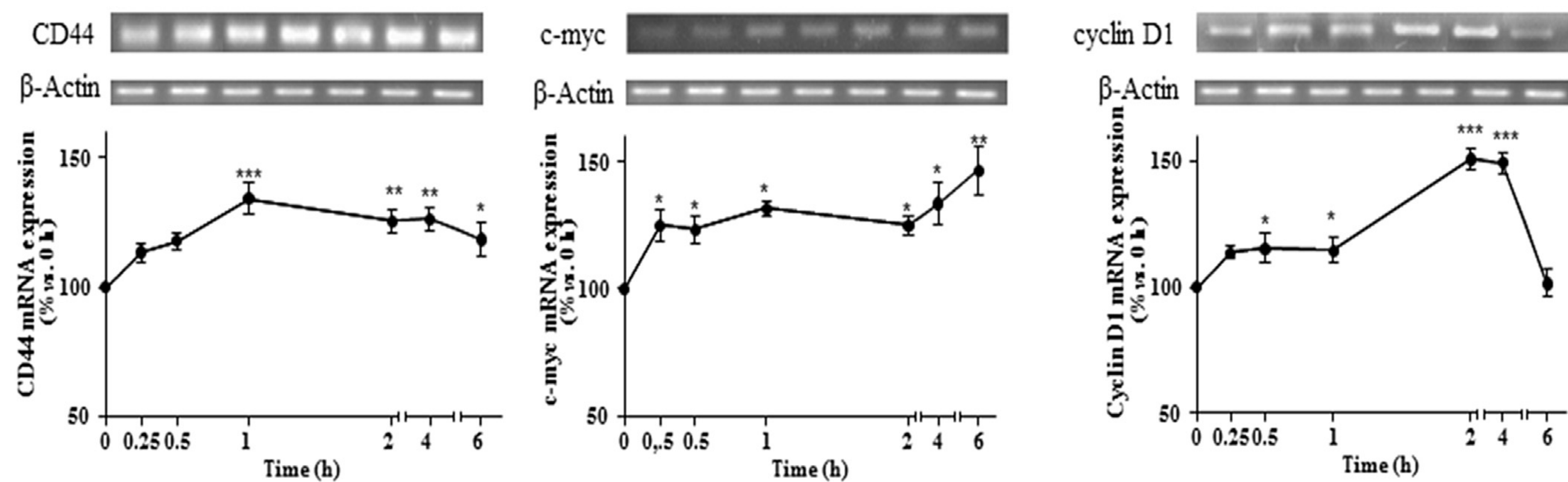
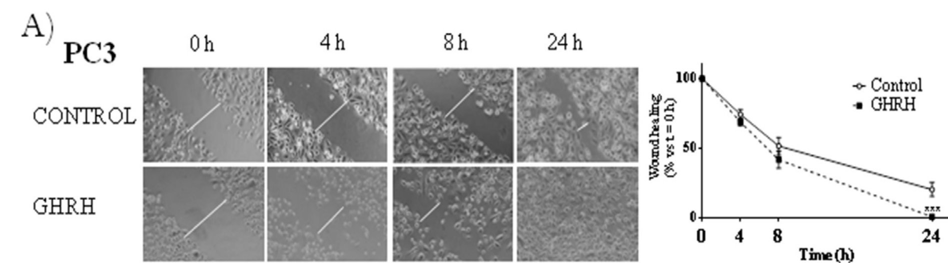
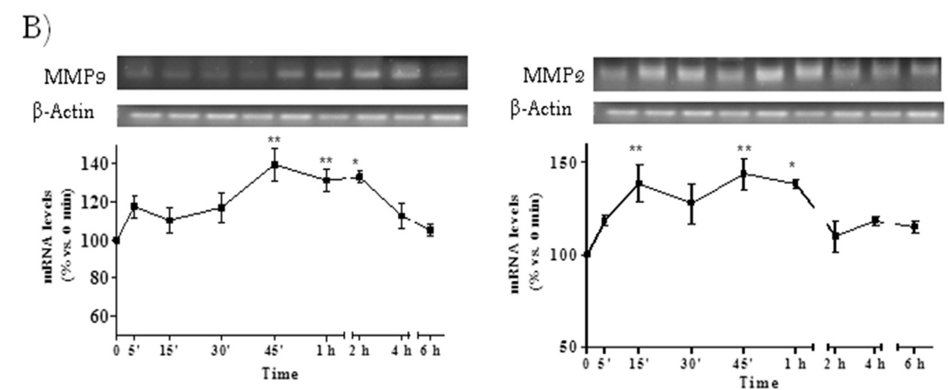
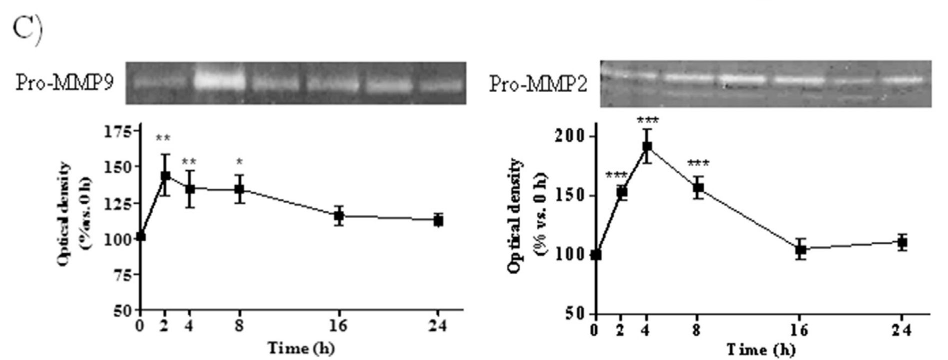


Figure 5







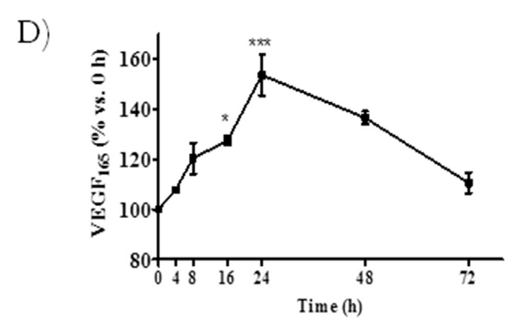


Figure 6