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# GRP78 Mediates Cell Growth and Invasiveness in Endometrial Cancer

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Recent studies have indicated that endoplasmic reticulum stress, the unfolded protein response activation and altered GRP78 expression can play an important role in a variety of tumors development and progression. Very recently we reported for the first time that GRP78 is increased in endometrial tumors. However, whether GRP78 could play a role in the growth and/or invasiveness of endometrial cancer cells is still unknown. Here we report that the silencing of GRP78 expression affects both cell growth and invasiveness of Ishikawa and AN3CA cells, analyzed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell migration assay, respectively. At variance with Ishikawa cells, AN3CA cells showed, besides an endoplasmic reticulum, also a plasma membrane GRP78 localization, evidenced by both immunofluorescence and cell membrane biotinylation experiments. Intriguingly, flow cytometry experiments showed that the treatment with a specific antibody targeting GRP78 C-terminal domain caused apoptosis in AN3CA but not in Ishikawa cells. Induction of apoptosis in AN3CA cells was not mediated by the p53 pathway activation but was rather associated to reduced AKT phosphorylation. Interestingly, immunofluorescence analysis evidenced that endometrioid adenocarcinoma tissues displayed, similarly to AN3CA cells, also a GRP78 plasma membrane localization. These data suggest that GRP78 and its plasma membrane localization, might play a role in endometrial cancer development and progression and might constitute a novel target for the treatment of endometrial cancer.

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Endometrial cancer (EC) is the most common malignancy of the female genital tract, and overall the endometrium is the fourth most frequent cancer site, accounting for 6% of all cancers in women (Bray et al., 2005; Di Cristofano and Hedrick Ellenson, 2007). Atypical endometrial hyperplasia (AEH) is felt to be a precursor of lesions and it may progress, over time, to EC in 5–25% of patients (D'andrilli et al., 2012). In addition, AEH is associated with a coexisting EC in approximately 20% of patients (Masciullo et al., 2010). Risk factors for endometrial carcinoma include long-term unopposed estrogen therapy, tamoxifen therapy, polycystic ovarian syndrome, estrogen-producing tumors, a history of nulliparity or infertility, irregular menstrual cycles, early age at menarche, late age at menopause, obesity, diabetes mellitus, and hypertension (Nyholm et al., 1993; Kaaks et al., 2002). An increasing number of studies have recently reported the activation of the Unfolded Protein Response (UPR), in response to ER stress, in various tumors (Steiner et al., 2003). The endoplasmic reticulum (ER) is an essential organelle for the synthesis and folding of secretory and membrane proteins. When the protein load exceeds the folding capacity of the ER, the cells trigger the UPR, which activates the PERK, IRE1/XBP-1, and ATF6 signaling pathways as protective measures, resulting in general translational

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attenuation, up-regulation of chaperones and folding enzymes, and enhanced ER-associated degradation (ERAD) of malfolded proteins (Ma and Hendershot, 2004). Depending on the severity of ER stress, the UPR can result in cell death through the activation of apoptotic pathways specifically mediated by the ER, as well as coupling with the mitochondrial pathways (Ma and Hendershot, 2004; Li et al., 2006). A major UPR target is the glucose-regulated protein 78 (GRP78), which has important roles in protein folding and assembly, ER Ca2+ binding, and controlling the activation of transmembrane ER stress sensors (Lee, 2001). Recent studies have indicated a potential role for the UPR activation and altered GRP78 expression and function in tumor development and progression (Zhang et al., 2006). Often tumor cells are confronted with oxygen deprivation and nutrient stress, even with extensive angiogenesis (Zhang et al., 2006). Such stresses can lead to the accumulation of inappropriately processed proteins and sensitize normal cells to apoptosis more readily when compared with cancer cells with elevated GRP78 expression (Jamora et al., 1996; Liu et al., 1998; Reddy et al., 1999). In multiple tumor types including prostate, lung, stomach, breast, gastric and epidermoid carcinoma, GRP78 overexpression confers also resistance to a wide variety of chemotherapeutic agents, and knockdown of GRP78 sensitizes tumor cells to drug treatment (Bernstein et al., 1999; Koomagi et al., 1999; Fernandez et al., 2000; Reddy et al., 2003; Daneshmand et al., 2007; Zheng et al., 2008). In addition, recent studies have established that GRP78 can be located in compartments outside the ER, including the cell surface, where it can bind different molecular partners and exert additional effects on cell growth and signaling of different cancer cells (Li and Lee, 2006; Kern et al., 2009; Ni et al., 2011). Hence, given the importance of GRP78 in cancer cell survival, it might represent a prime target for anticancer agents. Inhibitors of GRP78 could be used in combination with standard therapeutic agents to enhance drug efficacy and possibly eliminate residual resistant tumor cells (Andersson et al., 2010). We have recently described for the first time the presence of ER stress and GRP78 overexpression in endometrial cancer tissues (Bifulco et al., 2012). In the present study we evaluated the role of GRP78 in cell growth and invasiveness of endometrial cancer cells. We also verified if GRP78 might be localized on the cell surface of both endometrial cancer tissues and cells and whether this localization might play a role in endometrial cancer cells growth and survival.

## Methods

### Clinical and pathology

Specimens were composed of normal endometrium, atypical endometrial hyperplasia, and endometrial carcinoma. They were collected from the Department of Obstetrics and Gynecology of University of Naples "Federico II." All specimens were examined and tumors were histologically graded by an expert gynecological pathologist (LI) according to the guidelines of the International Federation of Gynecology and Obstetrics. Patients who were undergoing a total abdominal hysterectomy for benign conditions, were used as controls. Formalin-fixed, paraffin-embedded tissues from the specimens were selected. Representative slides of each tumor were stained with hematoxylin and eosin. Images were captured by a digital camera Axiocam color (Zeiss, Gottingen, Germany).

### Cell lines and reagents

The human endometrial cancer cell lines used were Ishikawa (kindly provided by Prof. S. Catalano, Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Italy), derived from well-differentiated endometrial adenocarcinoma and AN3CA (kindly provided by Dr. S. Petrosino, Institute of Biomolecular Chemistry of C.N.R., Pozzuoli, Italy), derived from undifferentiated endometrial adenocarcinoma. Ishikawa cells were grown in DMEM supplemented with 10% fetal bovine serum and 300 mM L-glutamine, in a humidified atmosphere with 5% $CO_2$. AN3CA were grown in MEM supplemented with 10% fetal bovine serum (FBS) and 300 mM L-glutamine, in a humidified atmosphere with 5% $CO_2$. DMEM, MEM, L-glutamine, FBS, and G418 were from Lonza (Verviers, Belgium). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye and tunicamycin were purchased from Sigma (St. Louis, MO). Transwell plates were from Corning (Acton, MA). The anti-GRP78 antibody recognizing the N-terminal domain of the protein has been previously described (Bifulco et al., 2012), the anti-GRP78 recognizing the C-terminal domain of the protein, anti-β-actin, anti-p53, anti-PARP, anti-cleaved caspase 9, anti-14-3-3, anti-AKT, anti-p-AKT, anti-ERK1/2 and anti-p-ERK1/2 antibodies were from Santa Cruz Biotechnologies (Dallas, TX). The Cell Surface Protein Isolation Kit and the Enhanced chemiluminescence Western blotting detection reagents were purchased from Pierce Biotechnology (Rockford, IL).

### Sh-RNA targeting

To inhibit expression of endogenous GRP78, cells have been stably transfected with either constructs containing short hairpin (sh)-RNA specific to human GRP78 or a scrambled sh-RNA with no homology to any known human mRNA, as negative control (Qiagen, Valencia, CA). Positive cells have been then selected by treating cells with 600 μg/ml G418.

### Kinetics of cell proliferation and determination of cell growth

Untransfected cells, cells stably transfected with a construct carrying a scramble sequence (Scr) or a sequence specific to human GRP78 (sh) were seeded at a density of $2 \times 10^4$ cells in 12-well plates in 1 ml of culture medium supplemented with 10% FBS. After 24, 48, 72, or 96 h, non-adherent cells were removed by gentle washing with PBS whether adherent cells were detached by trypsin treatment and counted using a standard counting chamber Neubauer.

### MTT assay and Western blot

In vitro proliferation was assessed with tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, 1,000 cells of each cell type (transfected or parental) were plated per well onto 96-well microtiter plates in medium with 10% FBS. One plate was developed immediately after cells had adhered (at approximately 4 h), and other plates were developed every 24 h for 4 days. Assays were done by incubating each plate with 20 μl of MTT substrate for 2 h followed by removal of medium and addition of 200 μl of dimethylsulfoxide. Plates were red at a wavelength of 570 nm. For Western blot assays, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested in Laemmli buffer (with β-mercaptoethanol) containing a mixture of phosphatase inhibitors (0.5 mM sodium vanadate, 2 mM sodium pyrophosphate, 5 mM β-glycerolphosphate, and 50 mM sodium fluoride) to prevent postlysis dephosphorylation. Western blots were carried out as previously reported (Bifulco et al., 2012). Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image J program (developed at the U.S. National Institutes of Health; available at http://rsb.info.nih.gov/nih-image/).

### Wound healing assay

Cells ($1 \times 10^6$ per well) were seeded in six-well plates and allowed to adhere for 24 h. Confluent monolayer cells were scratched by a 200 μl pipette tip and then washed three times with PBS to clear cell debris and suspension cells. Fresh medium was added, and the cells were allowed to close the wound for 48 h. Photographs were taken at 0, 24, and 48 h at the same position of the wound.

### Transwell migration assay

Cells ($2 \times 10^5$) were resuspended in 200 μl of serum-free medium and seeded on the top chamber of the 8 μm pore, 6.5 mm polycarbonate transwell filters. The full medium (600 μl) containing 10% FBS was added to the bottom chamber. The cells were allowed to migrate for 24 h at 37°C in a humidified incubator with 5% $CO_2$. The cells attached to the lower surface of membrane were fixed in 4% paraformaldehyde at room temperature for 30 min and stained with hematoxylin, and the number of cells on the lower surface of the filters was counted under the microscope. A total of 5 fields were counted for each transwell filter.

### Immunofluorescence

Tissues for immunofluorescence (IF) were treated as previously described (Calì et al., 2012). Deparaffinization, hydration and antigen retrieval of tissue 4 μm sections were performed by means of wax capture (W-CAP) antigen retrieval solution, pH 6.0, according to manufacturer's instructions (Bio-Optica, Milan, Italy). Tissue sections were incubated o.n. at 4°C with the home made GRP78 anti-serum diluted 1:250 in blocking buffer. AN3CA and Hishikawa cells grown on glass coverslips were fixed IHC Zinc fixative (BD Pharmingen, San Diego CA) at 4°C for 48 h and than treated as described (Gentile et al., 2004; Calì et al., 2012) without any permeabilization. Nuclei were stained with the DNA intercalator DRAQ5 5 μM (Alexis Corporation, Lausen, Switzerland), present in the PBS with 50% Glycerol mounting medium. Immunofluorescence analysis was performed at a confocal laser scanning microscope LSM 510 Meta (Zeiss). The lambda of the argon ion laser was set at 488 nm, that of the HeNe laser was set at 633 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488 and by 615 long pass filter for DRAQ5. Double staining immunofluorescence images were acquired separately in the green and infrared channels at a resolution of 1024 × 1024 pixels, with the confocal pinhole set to one Airy unit, and then saved in TIFF format. Fluorescence intensity analysis was performed on a Macintosh computer using the public domain NIH Image J program (developed at the U.S. National Institutes of Health; available at http://rsb.info.nih.gov/nih-image/).

### Biotinylation of cell surface proteins

Biotinylation of cell surface proteins was performed using the Cell Surface Protein Isolation Kit (Pierce Biotechnology). Briefly, four T75 flasks of AN3CA cells were grown to 95% confluency, washed twice with ice-cold PBS and incubated in 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin for 30 min at 4°C with rocking. Following saturation with quenching solution, the cells were scraped, pelleted at 500 g for 3 min, and washed several times with TBS. Cells were lysed in 500 μl of lysis buffer for 30 min on ice with vortexing every 5 min. The lysates were centrifuged at 10,000*g* for 2 min at 4°C, and the biotinylated proteins were isolated from the cleared supernatant by binding to immobilized NeutrAvidin slurry for 60 min at room temperature with rotation. The slurry was washed four times with wash buffer containing protease inhibitors, and the biotinylated proteins were solubilized in 400 μl of 4× SDS–PAGE sample buffer (50 mM Tris, pH6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 50 mM DTT) for 60 min at room temperature with rotation. As a control, total cell lysates were collected in SDS–PAGE sample buffer. Immunoblot analysis was used to identify target proteins of interest in both total and cell surface lysates. Densitometric analysis was performed on a Macintosh computer using the public domain NIH Image J program (developed at the U.S. National Institutes of Health; available at http://rsb.info.nih.gov/nih-image/).

### Measurement of apoptosis

Apoptosis was assessed by staining of cell membrane-exposed phosphatidylserine with fluorescein isothiocyanate-conjugated Annexin V according to the manufacturer's description (BD Pharmingen). Samples were analyzed by flow cytometry using a FACSCalibur (Beckman Instruments, Fullerton, CA), equipped with CellQuest Analysis Software.

## Results

### The attenuation of GRP78 expression inhibits proliferation of endometrial cancer cells

GRP78 is reported to play a role in the growth of different tumors. Therefore, we hypothesized that GRP78 might play a role also in endometrial cancer cells growth. To explore the role of GRP78 in the regulation of cell growth in endometrial cancer, we examined the proliferation rate of both AN3CA and Ishikawa cells untransfected or stably transfected with vectors containing either a scramble short hairpin sequence (Scr) or short hairpin sequences specific to GRP78 (Sh1-4). As shown in Figure 1, at variance with the scramble sequence, all the short hairpin sequences targeting GRP78 were able to decrease GRP78 protein level in both AN3CA (A) and Ishikawa cells (B), albeit with different efficacy. Interestingly, cell proliferation, evaluated by proliferation curves analysis, of both AN3CA (C) and Ishikawa cells (D) transfected with Sh was significantly decreased when compared to untransfected cells or cells transfected with the Scr sequence. Analysis of cell viability performed by MTT confirmed these observations in both AN3CA (E) and Ishikawa cells (F), suggesting that GRP78 might play a role in endometrial cancer cells growth.

### GRP78 knockdown reduces endometrial cancer cells migration and invasion

Next, since it has been reported that GRP78 is involved in the invasion and metastatic process of tumor cells, we examined whether Grp78 also participates in the malignant phenotypes of cell migration and invasion of endometrial cancer cells. To this aim, we performed wound healing and transwell migration assays on both AN3CA and Ishikawa cells untransfected or stably transfected with vectors containing either the scramble short hairpin sequence (Scr) or a short hairpin sequence specific to GRP78 (Sh). One of the most effective vector in downregulating GRP78 expression was used (Sh1 and Sh3 in AN3CA and Ishikawa cells, respectively). Monolayer cultures were wounded by a micropipette tip and cell migration toward the wounded area was observed. As shown in Figure 2, although the two different lines of cells migrated at different rates, the Sh transfectants in each line migrated slower toward to the wounded area compared with the Scr transfectants. At 48 h, the wounded area was almost completely covered by Scr-transfected cells, whereas the Sh-transfected cells were still moving toward into the area. Knockdown of Grp78 thus reduces migration, suggesting a positive regulation of this protein in cell migration. For the invasion assay, transfected

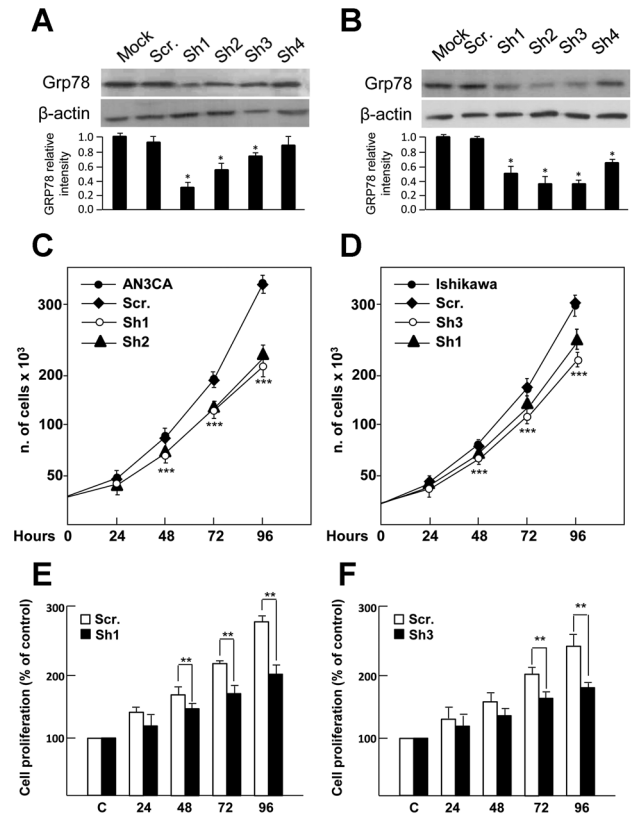


**Fig. 1. The attenuation of GRP78 expression inhibits proliferation of endometrial cancer cells. Western blots of total protein extracts from AN3CA (A) or Ishikawa cells (B) untransfected or stably transfected with a construct carrying a scramble sequence (Scr.) or four different sequences specific to human GRP78 (Sh1-4). Filters were probed with antibodies against GRP78 (home-made antibodies) and β-actin. Data represent mean values ± SD of triplicate samples of three independent experiments. * indicates a *P*-value <0.05. AN3CA (C) or Ishikawa cells (D) untransfected or stably transfected with a construct carrying a scramble sequence (Scr.) or two different sequences specific to human GRP78 were plated at a density of $2 \times 10^4$ cells. After 24, 48, 72, and 96 h, cells were counted using a Neubauer chamber. Data represent mean values ± SD of triplicate samples of three independent experiments. *** indicates a *P*-value <0.001. AN3CA (E) or Ishikawa cells (F) untransfected or stably transfected with a construct carrying a scramble sequence (Scr.) or a short hairpin sequence specific to human GRP78 (Sh3 or Sh1 respectively), were seeded at a density of $5 \times 10^3$ cells in a 96 well plate and cell viability was measured 24, 48, 72, and 96 h later using the MTT assay. Values represent the mean absorbance at 540 nm ± SD of triplicates of three independent experiments. White bars represent cells transfected with a construct carrying a scramble sequence (Scr.); black bars represent cells transfected with the short hairpin specific to human GRP78. A value of 100% was given to the cell number at time 0. ** indicates a *P*-value <0.01.**

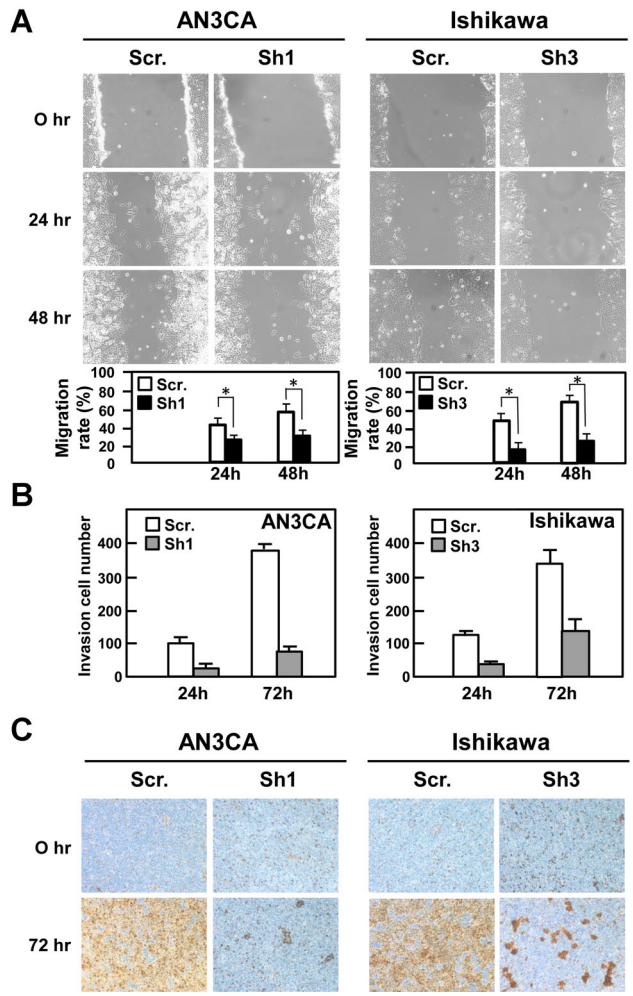


**Fig. 2. GRP78 knockdown reduces endometrial cancer cells migration and invasion. A: AN3CA or Ishikawa cells ($1 \times 10^6$ per well) transfected either with a construct carrying a scramble sequence (Scr.) or a short hairpin sequence specific to human GRP78 (Sh1 or Sh3 respectively), were seeded in six-well plates and allowed to form a cell monolayer for 24 h. Cell layers were wounded with a micropipette tip and then incubated in fresh culture medium for up to 48 h. Cell migration toward the wounded area was observed, photographed and measured. Results were obtained from three separate experiments; *$P < 0.05$. B: AN3CA or Ishikawa cells ($2 \times 10^5$ per well) transfected either with a construct carrying a scramble sequence (Scr.) or a sequence specific to human GRP78 (Sh1 or Sh3 respectively), were seeded in the upper chamber of a 24-well plate as described in the Materials and Methods Section. The number of cells migrating through the lower chamber were determined after 24 and 72 h. Each experiment was done in triplicate. C: A representative experiment of (B).**

cells were seeded in the upper chamber of transwell plates. The number of cells invading the lower chamber was determined after 24 and 72 h. As shown in Figure 2B,C, significant reduction of Sh-transfected cells were found invaded to the lower chamber compared with control Scr-transfected cells at both time point. Thus, knockdown of Grp78 expression suppresses the invasion ability of endometrial cancer cells.

### AN3CA cells express cell surface GRP78

A number of studies have identified GRP78 on the surface of a variety of human cancer cells (Mintz et al., 2003; Arap et al., 2004; Gonzalez-Gronow et al., 2006; Misra and Pizzo, 2007, 2010a, Misra et al., 2009, 2011). To determine if GRP78 is similarly expressed on the surface of endometrial cancer cells, cell surface proteins were isolated using a biotin-based technique followed by immunoblotting with anti-GRP78 antibodies. GRP78 was identified in both total cell lysates (t-GRP78) and on the cell surface of AN3CA cells (s-GRP78), while it was barely detectable on the surface of Ishikawa cells (Fig. 3A). GRP78 expression and surface localization were significantly increased following tunicamycin treatment (Fig. 3A) in both Ishikawa and AN3CA cells. Consistent with these biochemical findings, confocal microscopy observations

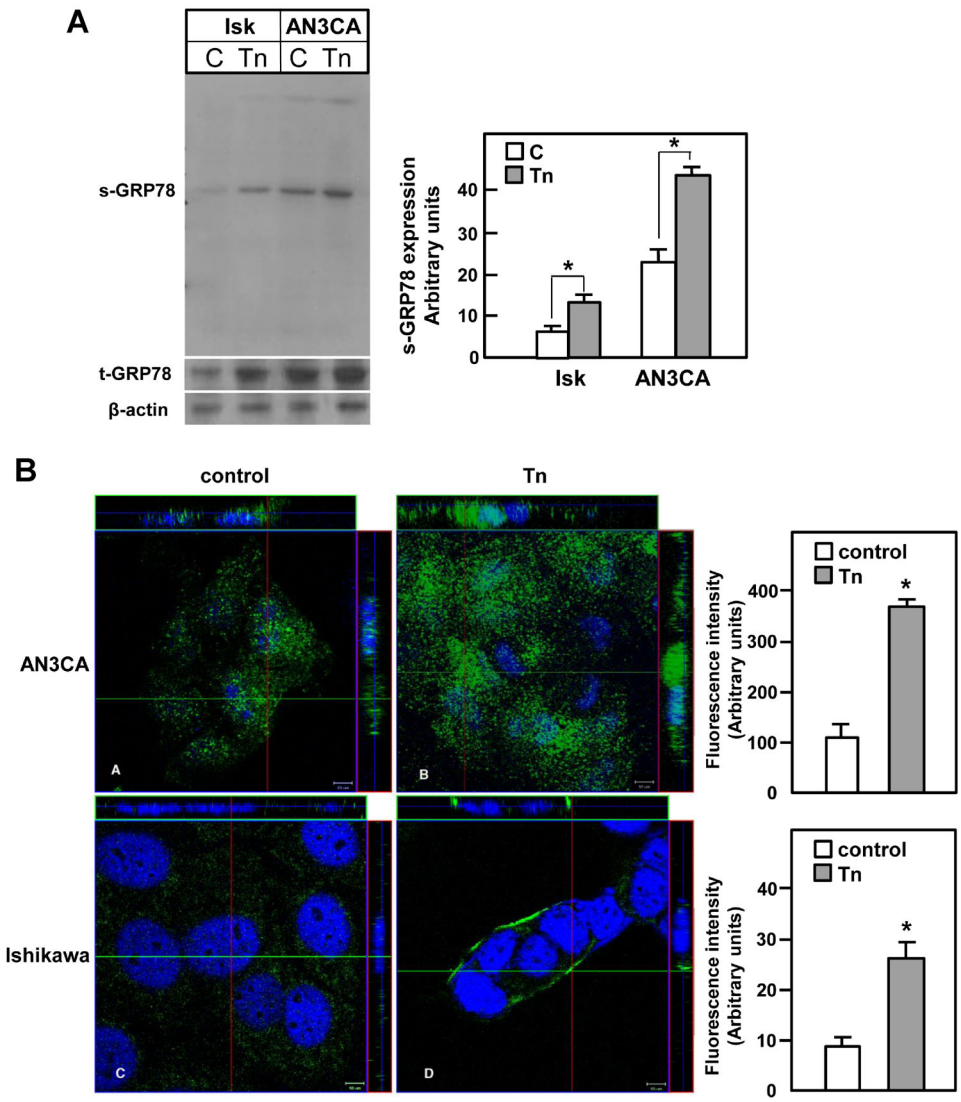


**Fig. 3. GRP78 localizes on the cell surface of endometrial cancer cells and ER stress further promotes its cell surface localization. A: Detection of cell surface GRP78 expression in Ishikawa and AN3CA cells untreated (C) or treated (Tn) with 1 μg/ml tunicamycin for 24 h. Cells were biotinylated and biotinylated proteins were isolated as described in the Materials and Methods Section. Representative Western blots are shown. s-GRP78 and t-GRP78, surface and total intracellular GRP78, respectively. β-Actin served as loading control. The graph represent the results of three independent experiments. *$P < 0.05$. B: AN3CA and Ishikawa cells were not treated (A and C, respectively) or treated (B and D, respectively) with 1 μg/ml tunicamycin (Tn) for 24 h. Non-permealized cells were fixed and stained by indirect immunofluorescence as described in the Materials and Methods Section using the GRP78 home made antiserum. Nuclei (in blue) are counterstained with the DNA intercalator DRAQ5. Section from confocal Z-stack images shows the increase of GRP78 staining on the plasmamembrane after Tn treatment. The green and red lines indicate the locations of the front and side views respectively. Scale bar 10 μm. Quantification of fluorescent intensity of Grp78 staining is also shown. The graph represent the results of three independent experiments; *$P < 0.05$.**

confirmed the presence of GRP78 on the cell surface of AN3CA cells (Fig. 3B, part A) but not on that of Ishikawa cells (Fig. 3B, part C). As observed in the membrane biotinylation studies, tunicamycin was able to markedly increase GRP78 membrane localization in both AN3CA and Ishikawa cells (Fig. 3B, part B and D, respectively).

### An antibody recognizing the C-terminal domain of GRP78 induces apoptosis of AN3CA but not Ishikawa cells

Treatment with antibodies against the carboxy-terminal domain of GRP78 has been reported to profoundly inhibit prosurvival signaling pathways in prostate cancer cells that display cell surface localization of GRP78 (Misra and Pizzo, 2007, 2010a; Misra et al., 2009 De Ridder et al., 2012). We therefore speculated whether these antibodies may affect the growth and/or viability of endometrial cancer cells in relationship with their GRP78 exposition on the plasma membrane. As shown in Figure 4A, following 48 h of treatment with the antibodies (+Ab), at variance with Ishikawa cells, AN3CA cells appeared to be slowed down in growth when compared to untreated cells (c). Moreover, an increased number of floating cells were easily observable in antibodies-treated cells (Fig. 4A). To confirm this observation, we performed MTT assays to analyze cell viability of treated versus

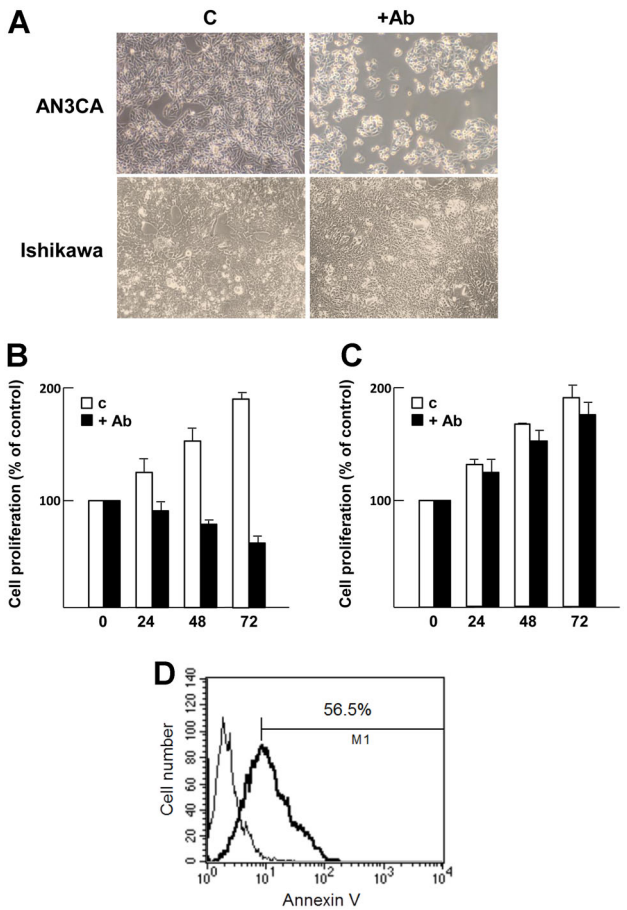


**Fig. 4. An antibody recognizing the C-terminal domain of GRP78 induces apoptosis of AN3CA but not Ishikawa endometrial cancer cells. A: AN3CA and Ishikawa cells were treated or not for 48 h with 200 μg/ml of an antibody recognizing the C-terminal domain of GRP78. Cells were observed by light microscopy and photographed. AN3CA (B) or Ishikawa (C) cells were seeded at a density of $5 \times 10^3$ cells per well in a 96 well plate and treated or not with 200 μg/ml of an antibody recognizing the C-terminal domain of GRP78. Cell viability was measured 24, 48, and 72 h later using the MTT assay. Values represent the mean absorbance at 540 nm ± SD of triplicates of three independent experiments. White bars represent untreated cells whether black bars represent cells treated with the antibody. D: AN3CA cells were treated or not with 200 μg/ml of an antibody recognizing the C-terminal domain of GRP78 for 72 h and subjected to annexin V staining as outlined in the Materials and Methods Section.**

untreated cells after 24, 48, and 72 h. As shown in Figure 4B, AN3CA cells treated with the antibodies (+Ab) displayed a significant decline in the percentage of proliferating cells when compared to untreated cells (Fig. 4B). At variance, Ishikawa cells did not show a significant difference in the proliferation rate of treated versus untreated cells (Fig. 4C). To understand if this might be due to an increase of cell death following the treatment, we performed flow cytometry analysis with Annexin V on AN3CA cells left untreated or treated for 72 h with the antibodies specific to the carboxy-terminal domain of GRP78. As shown in Figure 4D, antibodies-treated cells showed a high percentage of apoptotic cells (56.5%). These data suggest that cell surface GRP78 might play a role in the growth and survival of endometrial cancer cells.

### The knockdown of GRP78 and the treatment with antibody recognizing the C-terminal domain of GRP78 reduce prosurvival signaling in endometrial cancer cells

Apoptosis in AN3CA cells but not in Ishikawa cells, following antibodies treatment, was confirmed also by the cleavage of poly(ADP-ribose) polymerase (PARP) and caspase 9, hallmarks of apoptosis (Fig. 5). Since it has been described that ligation of cancer cell surface GRP78 with antibodies directed against its C-terminal domain can promote apoptosis in pancreatic cancer cells by up-regulating p53 (Misra and Pizzo, 2010b), we evaluated p53 levels in both AN3CA and Ishikawa cells following antibodies treatment. As shown in Figure 5, despite the fact that AN3CA cells express wild type p53 weather Ishikawa cells express a mutated inactive form of the protein, p53 protein levels did not increase significantly in both AN3CA and Ishikawa cells following GRP78 antibodies treatment, suggesting that the activation of apoptosis was p53-independent. Next, to understand the molecular mechanism/s involved in the induction of apoptosis caused by the antibody specific to the C-terminal domain of GRP78, we analyzed the phosphorylation levels of AKT and ERK proteins, known to be involved in prosurvival signaling. As shown in Figure 5, phosphorylation levels of AKT were significantly downregulated in antibodies treated AN3CA cells when compared to untreated cells. At variance, phosphorylation levels of AKT were unchanged in Ishikawa cells. Phosphorylation levels of ERK did not vary significantly following antibody treatment in both cell types (Fig. 5). These data suggest that GRP78 localization on the surface of endometrial cancer cells plays a role in endometrial cancer cells survival and that apoptosis induction is due by a p53-independent mechanism involving the inhibition of AKT prosurvival signaling pathway.

### GRP78 membrane localization is increased in the membrane of endometrial cancer tissues

Very recently we have reported for the first time that GRP78 is overexpressed in tissue specimens of endometrioid endometrial carcinomas (Bifulco et al., 2012). To address the question if GRP78 could be localized, besides on the surface of cultured endometrial cancer cells, also on the surface of endometrial tissues, we performed immunofluorescence studies on tissue specimens of normal endometrium (N), endometrial hyperplasia with atypia (HA) or endometrioid adenocarcinomas characterized by different grading (G1 or G2). As shown in Figure 6, in non-pathological tissue (A) anti-GRP78 antibodies stain prevalently epithelial cells and the staining appears to be confined prevalently to the perinuclear region, compatible with an ER localization, but also to the plasmamembrane. In the endometrial hyperplastic tissue characterized by atypia, the staining of GRP78 is slightly enhanced in the plasmamembrane (E). In the G1 (I) and G2 (M) carcinoma sections the staining of GRP78 protein is significantly increased in both the cytoplasm and the plasmamembrane region. These observations suggest that GRP78 membrane localization is increased in the membrane of endometrial cancer tissues.

## Discussion

Very recently we have described for the first time that ER stress is activated and GRP78 is overexpressed in endometrial cancer tissues (Bifulco et al., 2012). Therefore we hypothesized that GRP78 might play a role in endometrial cancer cells proliferation and/or invasiveness. In this study, we show that the inhibition of GRP78 expression by the use of specific shRNAs significantly decreases the growth of endometrial cancer cells. GRP78 has been implicated as an important player in

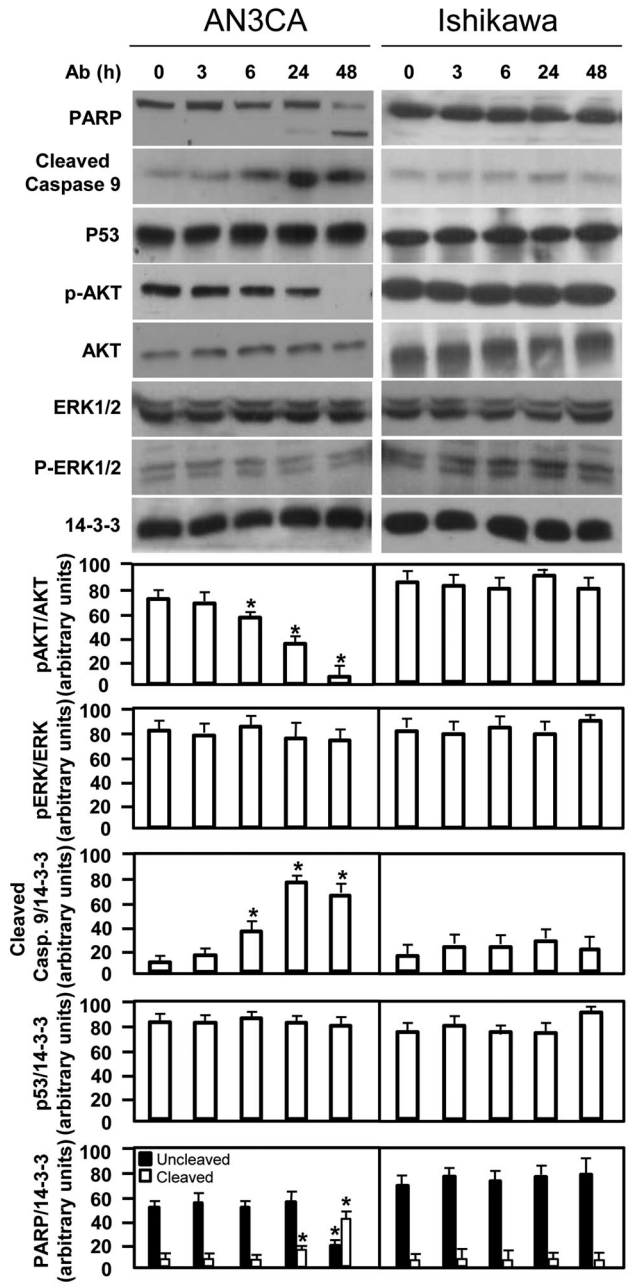


**Fig. 5. The treatment with antibodies recognizing the C-terminal domain of GRP78 reduces AKT prosurvival signaling in AN3CA but not in Ishikawa endometrial cancer cells. AN3CA and Ishikawa cells were left untreated or treated for the indicated times with 200 μg/ml of an antibody recognizing the C-terminal domain of GRP78. Cells were harvested, lysed as described in the Materials and Methods Section and 50 μg whole-cell lysates were immunoblotted using anti-PARP, anti-cleaved caspase 9, anti-AKT, anti-p-AKT, anti-ERK1/2, anti p-ERK1/2, anti-p53, and anti-14-3-3 antibodies. Graphs show densitometric analysis of the bands. Results were obtained by three separate experiments; *$P < 0.05$.**

cancer progression by its role in promotion of tumor cell proliferation and survival during ER stress that arises in the tumor microenvironment as a result of hypoxia and nutrient deprivation (Fu and Lee, 2006; Li, 2012). Increased expression of GRP78 has been indeed observed in breast, colon, prostate and melanoma cancer cell lines, as well as in ex vivo human primary and animal model tissues (Lee, 2007). In support of the notion that GRP78 is more critically needed for the survival of stressed cells such as cancer, heterozygous GRP78 mice with half of wild-type (WT) GRP78 level are comparable to WT siblings in growth and development. However, tumor progression is significantly impeded in these mice as exemplified by a longer latency period, reduced tumor size, and increased tumor apoptosis (Lee and Hendershot, 2006). This is consistent with earlier studies showing that GRP78 reduction in xenografts inhibited tumor formation and growth. Moreover, knockout of GRP78 in prostate epithelium has been reported to inhibit prostate cancer formation in mice (Fu et al., 2008). GRP78 may also be important for tumor metastasis because it is elevated in metastatic cancer cell lines and lymph node metastasis, and knockdown of GRP78 inhibits tumor cell invasion in vitro and growth and metastasis in xenografts models (Fu and Lee, 2006; Zhang et al., 2006). Here we show that GRP78 inhibition reduces the migration and invasion capability of endometrial cancer cells. The mechanism whereby GRP78 promotes growth and metastasis is just emerging. Part of this mechanism might be related to the localization of the protein. Although traditionally GRP78 is regarded as a lumen ER chaperone whose major function is to fold and process ER proteins, bind ER Ca2+ and maintain ER homeostasis, recent studies have established, indeed, that in specific cell types or when subjected to stress, GRP78 can be located in compartments outside the ER, including the cell surface, the cytosol, the mitochondria and the nucleus, and it can even be secreted (Kern et al., 2009; Ni et al., 2011; Ouyang et al., 2011). There is evidence for low constitutive surface expression of GRP78 on several cell types, including vascular endothelia (Jamora et al., 1996; Steiner et al., 2003). However, membrane GRP78 has been detected mainly on cancer cells where it can act as a multifunctional receptor, activating downstream pro-proliferative and anti-apoptotic signaling cascades including RAS/MAPK and PI3-kinase/Akt (Misra et al., 2005; Shani et al., 2008; Gonzalez-Gronow et al., 2009; Miharada et al., 2011). We used cell surface biotinylation to demonstrate the expression of GRP78 on the surface of endometrial cancer cells, a finding consistent with other cancer cells. Indirect immunofluorescence confirmed the presence of cell surface GRP78. Interestingly, AN3CA cells, deriving by an undifferentiated endometrial tumor, showed a GRP78 membrane localization that was further increased by treatment with tunicamycin, an ER stress inducer. At variance, Ishikawa cells, obtained by a well differentiated endometrial tumor showed a clear plasma membrane localization only upon stimulation with tunicamycin, suggesting that the amount of GRP78 in the plasma membrane might be related to the differentiation degree of endometrial tumors. To ascertain the latter correlation we performed immunofluorescence analysis of tissue samples representative of different stages of endometrial cancer progression. Interestingly we found that GRP78 protein expression tended to increase in tissue sections of endometrium characterized by hyperplasia with atypia, known to be a precancerous condition, when compared to normal tissues. The amount of GRP78 further increased in tissue samples of G1 and G2 endometrial tumors and GRP78 localization in the plasma membrane paralleled the increase of expression observed in pathological tissues, supporting the notion that upregulation of cell surface GRP78 might be part of a more aggressive phenotype (Misra et al., 2005). A commercial polyclonal antibody directed against the C-terminus of GRP78 was reported to induce apoptosis in melanoma cells (A375) and prostate cancer cells (1-LN and DU145), but not in another prostate cancer cell line, PC-3, where GRP78 expression was undetectable on the surface (Misra et al., 2011). The proposed mechanism is that this antibody leads to the upregulation of

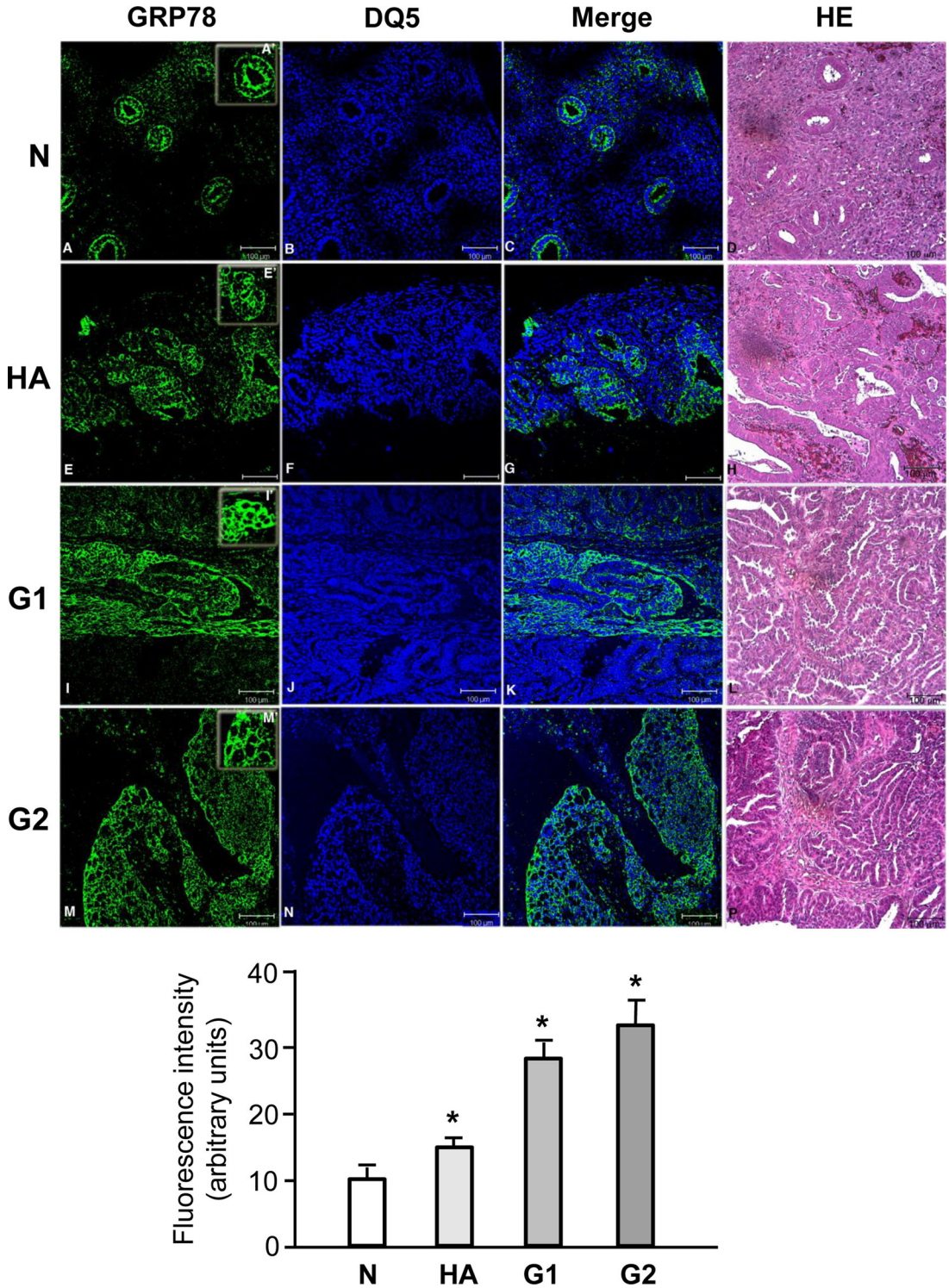


**Fig. 6. GRP78 localizes on the membrane of cells of pathological endometrial tissues. Confocal microscopy images of human endometrial tissue sections representative of normal endometrium (A–D), dysplastic endometrium with atypia (E–H), G1 (I–L), and G2 (M–P) endometrial tumors were immunostained with a polyclonal home made GRP78 anti-serum (A,E,I,M). Nuclei (in blue) are counterstained with the DNA intercalator DRAQ5 (B,F,J,N). Hematoxylin and eosin staining of the paraffin sections are also shown (D,H,L,P). Scale bar 100 µm. Fluorescence intensity measurements of Grp78 staining is also shown.**

p53, inhibition of NF-κB1 and NF-κB2 activation, and suppression of Ras/MAPK and PI3K/Akt signaling (Misra et al., 2005; Shani et al., 2008; Gonzalez-Gronow et al., 2009; Miharada et al., 2011). Since we observed that AN3CA cells display also a plasma membrane localization of GRP78, we treated cells with a commercial polyclonal antibody directed against the C-terminus of GRP78. Similarly to what described on melanoma and prostate cancer cells that display a plasma membrane localization of GRP78, viability of AN3CA cells treated with the antibody directed against the C-terminus of GRP78 was significantly decreased, when compared to that of untreated cells, and was associated to a marked high percentage of cells undergoing apoptosis. As expected, viability of Ishikawa cells, that did not display GRP78 on their surface, was not affected by the antibody treatment, indicating that cell surface expression of GRP78 in endometrial cancer cells is required for observing an effect of antibodies directed against the COOH-terminal domain of GRP78. Interestingly, at variance with Ishikawa cells, prosurvival PI3K/Akt signaling was significantly decreased in AN3CA cells. This is not surprising since AKT is constitutively activated in the majority of endometrioid endometrial cancers, due to PTEN loss and/or PI3K mutations (Slomovitz and Coleman, 2012). Activated AKT initiates a cascade of downstream signaling events, which promote cellular growth, metabolism, proliferation, survival, migration, apoptosis, and angiogenesis. Very recently it has been described that GRP78 contributes to cisplatin resistance of different endometrial cancer cell lines and that GRP78 expression contributes to chemoresistance through AKT activity (Gray et al., 2013). Downregulating GRP78 expression by the use of specific siRNA, was able, indeed, to decrease AKT activity and to sensitize endometrial cancer cells to apoptosis (Gray et al., 2013). We evaluated also the activation state of ERK, one of the key mediator of MAPK prosurvival signaling pathways, but we did not observe any significant alteration of its phosphorylation levels upon antibodies treatment in both apoptotic AN3CA and non apoptotic Ishikawa cells. Finally, at variance with apoptosis induced by the anti-GRP78 antibody in prostatic cancer cells, we did not observe significant variations of the p53 protein expression levels in AN3CA treated cells. However, it cannot be ruled out the involvement of other key mediators of the apoptotic pathway. This aspect is currently under investigation in our laboratory. In sum, we can conclude that down-regulating the levels of cellular GRP78 and/or targeting cell surface-associated GRP78, could be of major importance in endometrial cancer therapy.

## Statistical Procedures

Where not specified, data were analyzed with Statview software (Abacus Concepts, Piscataway, NJ) by one-factor analysis of variance. $P < 0.05$ was considered statistically significant.

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