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The role of Endoplasmic Reticulum stress and GRP78 in endometrial cancer

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LIST OF ABBREVIATIONS

AMPK: 5' adenosine monophosphate-activated protein kinase

ASK1: Apoptosis Signal Regulated Kinase

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

BCL2: B-cell lymphoma 2

BMI: Body Mass Index

CC: Compound C

CHOP: C/EBP homologous protein

EGCG: Epigallocatechin-3-gallate

eIF2α: Eukaryotic Initiation Factor 2

EMT: Epithelial mesenchymal transition

ER: Endoplasmic reticulum

ERAD: ER Associated Degradation

ERSE: ER stress response element

FBS : Fetal bovine serum

FIGO: International Federation of Gynecology and Obstetrics

GRP78: 78 kDa glucose-regulated protein

GRP94: 94 kDa glucose-regulated protein

HSP72: Heat Shock protein 72 kDa

HSP90 Heat Shock protein 90 kDa

IRE1: Inositol-Requiring Enzyme 1

JNK: c-Jun N-terminal kinase

MAPKKK: Mitogen-Activated Protein Kinase Kinase Kinase

NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

PARP: Poly (ADP-ribose) polymerase

PDI: Protein disulfide isomerase

PERK: Protein kinase RNA-like Endoplasmic Reticulum Kinase

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

SCR: Scramble Sequence

SERCA: Sarco/Endoplasmic Reticulum Ca2+-ATPase

ShRNA: short hairpin RNA

T2DM: Diabetes mellitus type 2

TGFβ: Tumor Growth Factorβ

TLR: Toll Like Receptor

TRAF2: TNF-Receptor-Associated Factor 2

UPR: Unfolded Protein Response

VEGF: Vascular Endothelial Growth Factor

XBP1: X-box Binding Protein 1

ABSTRACT

Endometrial cancer is the most common malignancy of the female genital tract. However, in spite of a huge advance in our understanding of endometrial cancer biology, therapeutic modalities haven't significantly changed over the past 40 years. 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. Our previous studies reported for the first time that GRP78 is increased in endometrial tumors.

In this study, we further analyzed the role of UPR and GRP78 in endometrial cancer progression. We found that GRP78 plays a role in endometrial cancer progression since its silencing attenuate both the growth and invasion of endometrial cancer cells. Interestingly, we also show that metformin, an antidiabetic drug with anticancer properties, is able to inhibit endometrial cancer cells growth and this is accompanied by the inhibition of GRP78 expression and upregulation of proapoptotic UPR genes such as ATF4 and CHOP. Finally, we describe that metformin affects β-catenin signaling, a frequently activated signaling pathway in endometrial cancer. These observations highlight the possibility that GRP78 might represent an intriguing therapeutic target of metformin action in the treatment of endometrial cancer.

1. BACKGROUND

1.1.Endoplasmic Reticulum Stress

In eukaryotic cells, the endoplasmic reticulum (ER) is an essential organelle for calcium storage and lipid synthesis. Furthermore, it is the site for synthesis, folding and post-translational modifications of cargo proteins[1].

The maturation of nascent proteins in the endoplasmic reticulum is monitored by multiple disulfide isomerases (eg. PDI), that catalyze the disulfide bonds formation, and a functional class of protein families, calcium-dependent "molecular chaperones" (as GRP78, GRP94 and calreticulin), which are associated with the newly synthesized proteins in order to prevent aggregation and enable their correct folding in both physiological and pathological conditions[2]. Therefore, the ER is a quality control system to ensure that only properly folded and functional proteins can be released from the ER and reach their final destination, while misfolded or inoperative proteins are retained in the ER and retrotranslocated in the cytosol for degradation by the 26S proteasome through the ER Associated Degradation (ERAD) machinery.

Numerous environmental, physiological and pathological insults, as well as nutrient fluctuations, can affect the ER protein-folding environment causing accumulation of misfolded proteins, a condition known as ER stress.

The formation of protein aggregates is toxic to cells and is the biochemical basis of many pathophysiological conditions such as ischemia, neurodegenerative diseases, diabetes and cancer[3].

To cope with the deleterious effects associated to ER stress, cells have evolved a system of homeostatic responses known as Unfolded Protein Response (UPR). This complex cellular response is mediated by the activation of three ER transmembrane proteins that act as sensors of ER stress: Pancreatic ER kinase (PERK), Activating Transcription Factor 6 (ATF6) and Inositol-Requiring Enzyme 1 (IRE1).

In the absence of ER stress, these proteins are maintained in an inactive state through their association with the chaperone molecule Glucose Regulated Protein 78 (GRP78). On the contrary, when ER stress and, thus, accumulation of misfolded proteins occurs, GRP78 dissociates from the three sensors determining their activation and inducing the UPR (Fig. 1).

The UPR, by attenuating the general translation process, upregulating the transcription of genes encoding for ER chaperones and folding enzymes and enhancing the degradation of malfolded proteins by the ERAD machinery tries to alleviate ER stress. Depending on the persistence and severity of ER stress, the UPR can ultimately result in cell death through the activation of apoptotic pathways specifically mediated by the ER, as well as coupling with the mitochondrial pathways[4].
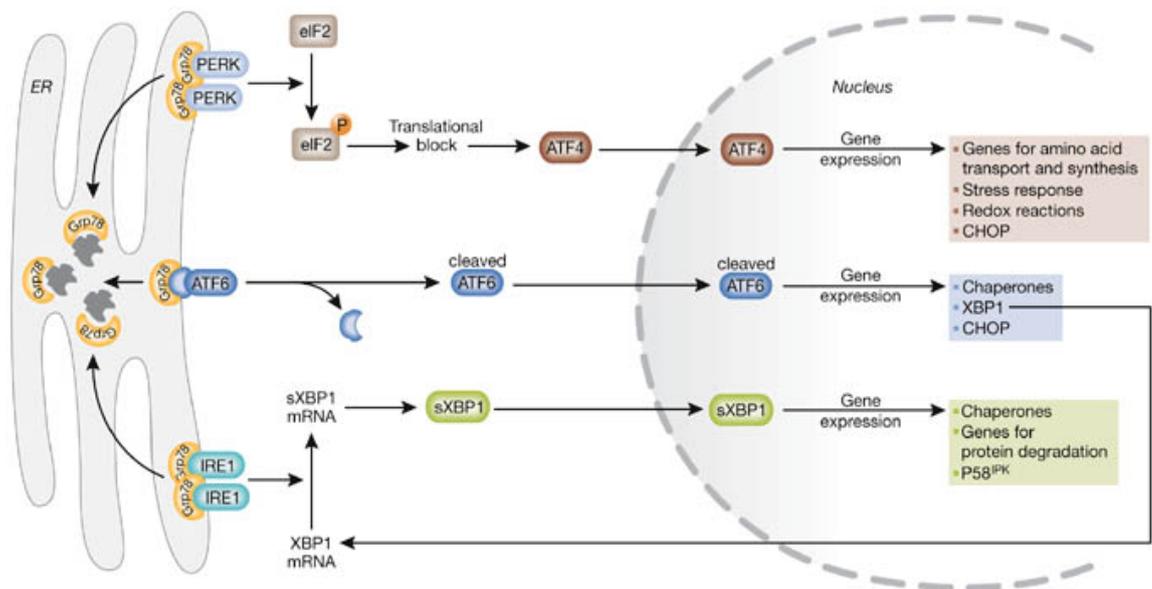
Fig. 1. The response to stress mediated by the UPR. *The GRP78 protein dissociates from the three ER stress sensors (ATF6, PERK and IRE1) causing their activation. PERK is the first to be activated followed by ATF6 while IRE1 is activated only later. ATF4 and ATF6 mediate transcription of CHOP, while IRE1 splices XBP1 mRNA (sXBP1) generating an active transcription factor that translocates to the nucleus activating the transcription of chaperones and P58IPK to restore the endoplasmic reticulum function[3].*

1.1.1. PERK

PERK is a type I transmembrane kinase which is activated by the dissociation of GRP78 by its luminal domain with subsequent dimerization and autophosphorylation of the protein.

Upon activation, PERK determines Ser51 phosphorylation of the translation initiation factor eIF2α (eukariotic translation initiation factor 2α), which regulates the attack initiator Met-tRNA to the ribosome, thus resulting in the inhibition of protein translation[5]. This event promotes cell survival by inhibiting further accumulation of nascent proteins in the ER.

Since eIF2α is the direct substrate of the kinase PERK the block of protein translation occurs almost immediately[6].

However, by means of still poorly known mechanisms, some mRNAs, such as that coding for the transcription factor ATF4 (activating transcription factor 4), is able to evade this translational block[4] (Fig. 1). ATF4 promotes cell survival via activation of genes involved in amino acid metabolism, redox reactions, and protein secretion[7].

ATF4 also activates the transcription of C/EBP homologous protein (CHOP; also known as DDIT3 and GADD153)[8], which is required for ER-stress-mediated apoptosis both in vitro and in vivo[9]. Initially, PERK activation represses CHOP transcription through miR-211 expression and histone methylation[10]. Under conditions of severe or chronic stress, constitutive PERK-mediated phosphorylation of eIF2α leads to apoptosis, as the prosurvival IRE1α–XBP1 and ATF6α pathways are attenuated[11,12]. It is likely that the particular response differs between cell types and environments on the basis of different 'thresholds' for ER stress tolerance of the cell. Although CHOP accumulation in the cell correlates with cell death, both ATF4 and CHOP mRNAs and proteins have short half-lives; therefore, a strong and chronic activation of PERK is necessary to increase steady state levels of CHOP to promote cell death[11]. Therefore, PERK activation promotes both adaptive as well as apoptotic responses depending on the severity of the stress.

1.1.2. ATF6

In mammals, the ATF6 sensor is present in two isoforms, called ATF6α (90 kDa) and ATF6β (110 kDa). ATF6 is a type II transmembrane glycoprotein, whose luminal domain is responsible for the detection of misfolded proteins. The cytoplasmic portion of ATF6 is able to act as a transcriptional factor in that it contains a DNA binding domain. Following its dissociation from GRP78, ATF6 translocates to the Golgi apparatus, where it undergoes a proteolytic cleavage at the level of its iuxta membrane domain, by the serine proteases S1P and S2P, generating an active transcription factor[13] (Fig. 1). ATF6 in its active form may therefore translocate into the nucleus, where it induces the transcription of target genes that possess in their promoter region ER stress response element/s (ERSE)[14]. Among the genes regulated by ATF6, have been identified those coding for some protein chaperones such as GRP78, GRP94 and PDI as well as other such GADD153/CHOP and the X-box binding protein 1 (XBP1). ATF6α mediates also the adaptive response to ER protein misfolding by upregulating the transcription of genes tha increase ER capacity and ERAD [15,16], whereas ATF6β may function as a repressor of ATF6α-mediated transcription and function[17]. At present, no genes have been identified that require ATF6β for their expression. PERK–eIF2α signaling facilitates ATF6α synthesis and trafficking to enhance ATF6α signaling[18]. To date, no substantial evidence supports a role for ATF6α in ER stress-induced apoptosis.

1.1.3. IRE1

IRE1 is a sensor with dual enzymatic activity, as it has both a serine-threonine kinase domain and endoribonuclease domain[19].

Under non-stress conditions, the proteins HSP90 and HSP72 bind the IRE1α cytosolic domain to maintain its stability[20,21], while GRP78 binds the luminal domain of IRE1α to prevent dimerization. Upon ER stress, unfolded and misfolded proteins bind and sequester GRP78, thereby releasing IRE1α for oligomerization, autophosphorylation and activation of its kinase and endoribonuclease activities[22,23].

IRE1 catalyzes the unconventional splicing of 26 nucleotides from the mRNA encoding, the X-Box Binding Protein-1(XBP1)[15], determining a frame-shift and generating protein with the original N-terminal DNA-Binding Domain and an additional transactivation domain at the C-terminus[24,25]. The resulting protein (sXBP1) encodes a transcription factor stable and active. sXBP1 translocates to the nucleus where it determines the transcription of genes encoding for the ER chaperons, ERAD components and P58IPK, a member of the family of heat shock protein 40 (HSP40IPK)[26]. P58IPK is able to bind to PERK and, by means of a negative feedback mechanism, removes the PERK-mediated translational block[27]. Forced XBP1s expression inhibits CHOP expression, thereby promoting cell survival[28].

Although the activation of IRE1 and splicing of XBP mRNA events appear to be more involved in cell survival, since they determine the induction of the transcription and synthesis of GRP78, GRP94, and P58IPK, it has been demonstrated that they can play a role in apoptosis activation under irreversible ER stress conditions[29]. Such a pro-apoptotic effect of IRE1 is mediated by the activation of the JNK kinase[30]. In particular, IRE1 is be able to recruit the adapter molecule TNF-receptor-associated factor 2 (TRAF2) and to activate Apoptosis Signal Regulated Kinase (ASK1), a Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK), which can transmit the death signal to the MAPKs JNK and p38[31]. Once activated, JNK is responsible for the phosphorylation of bcl2 abolishing its anti-apoptotic activity. Furthermore, it is also able to determine the phosphorylation of the pro-apoptotic BH3/BIM protein, enhancing its pro-apoptotic effect[32]. Thus, in presence of ER stress, activation of IRE1 play a critical role in the initiation of pro-apoptotic signals, while the activation of PERK and ATF6 sensors appears to precede the activation of IRE1 in the attempt to resolve the situation to the ER stress. If the stress persists, the PERK and IRE1 pathways can converge, mediating the induction of apoptosis through a mutual enhancement.

1.2. ER Stress and Cancer

Recently, much research in oncology has revealed that ER stress and UPR activation play a crucial role during the process of carcinogenesis. Therefore, they were recognized as critical processes in the biology of cancer cells as responsible for the maintenance of the malignant phenotype and acquisition of chemoresistance[1]. Tumor cells, which proliferate rapidly, require indeed an increased activity by the ER, to facilitate the folding, assembly and transport of secretory and membrane proteins. In contrast, normal cells are not subject to ER stress and the UPR is mainly in an inactive state (Fig. 2A). At the same time, tumor cells experience unfavorable conditions, due to an inadequate blood supply, such as hypoxia and nutrient deprivation. An inadequate supply of glucose influences both the process of protein glycosylation and production of ATP molecules, contributing to the accumulation of incorrectly folded proteins resulting in ER stress (Fig. 2B). Consequently, the UPR activation has been observed in several tumors types[1].

UPR activation protects cancer cells from stress-induced cell death[33-35]. Acute UPR activation enhances the protein folding capacity to meet the need for increased protein synthesis, which benefits cancer cell survival. Where chronic ER stress kills normal cells, tumor cells use strategies that neutralize apoptosis when challenged with ER stress. In response to chronic stress, normal cells usually attenuate the IRE1α–XBP1 and ATF6α pathways, so that the apoptotic signals dominate[36]. Some cancer cells, however, exhibit constitutive activation of the IRE1α–XBP1 pathway[37,38] or overexpression of GRP78 [39,40], which are anti-apoptotic.

During tumorigenesis, the marked increase in the levels of GRP78 and other chaperones of the ER by the UPR can improve the ER ability to folding and help to maintain homeostasis. In addition, the anti-apoptotic properties of GRP78 can counteract the cell death signals that are still functional in cancer cells. The maintenance of a chronic level of ER stress, through the expression of GRP78 and ATF6 would, therefore, be particularly effective in promoting growth and tumor progression[41].

Under normal conditions, GRP78 is localized in the ER lumen, but upon overexpression, as described in many human cancers, it becomes detectable on the cell surface[42]. GRP78 at the plasma membrane[43] can function as a multi-functional receptor[44], amplifying the PI3K/AKT pathway and promoting both proliferation and cell survival. It has been described that GRP78 in membrane can form a complex with Cripto protein, contributing to the inhibition of the TGF-β pathway, thus promoting the proliferation of cancer cells[45]. GRP78 expression not only correlates with cancer cell proliferation and histological grade but also correlates with response to therapies and prognosis[46].

ER stress and the UPR activation may promote tumorigenesis in various ways (Fig. 3).

For example, ER stress promotes angiogenesis by stimulating both expression and secretion of VEGF (Vascular Endothelial Growth Factor)[47,48] in both tumor and endothelial cells.

In particular, ATF4 and XBP1s can directly bind to the VEGFA promoter to initiate VEGFA transcription. VEGFA mRNA stability is also increased in response to UPR activation, via activation of AMPK[48].

ER stress and UPR activation, aside from the effects on tumor cell survival and proliferation can promote cancer progression also through the activation of inflammatory responses.

All three UPR pathways can indeed lead to the activation of nuclear factor-κB (NF-κB), a master transcriptional regulator of pro-inflammatory pathways[49].

Recent studies also suggest a role for ER stress in promoting macrophage activation and inflammation in the microenvironment of the tumor[50].

Macrophages grown in conditioned medium from tumor cells with ER stress become, indeed, stressed and also activated enhancing the proinflammatory response of tumor cells, thus favoring tumor progression (Fig. 3). ER stress in macrophages promotes the type M2 macrophage phenotype[51] that in turn supports tumor growth. In addition, ER stress-induced expression of CHOP, in combination with TLR agonists, enhances dendritic cell expression of IL-23[52] that favours development of T helper 17 (TH17) cell-mediated inflammation and tumor growth[53].
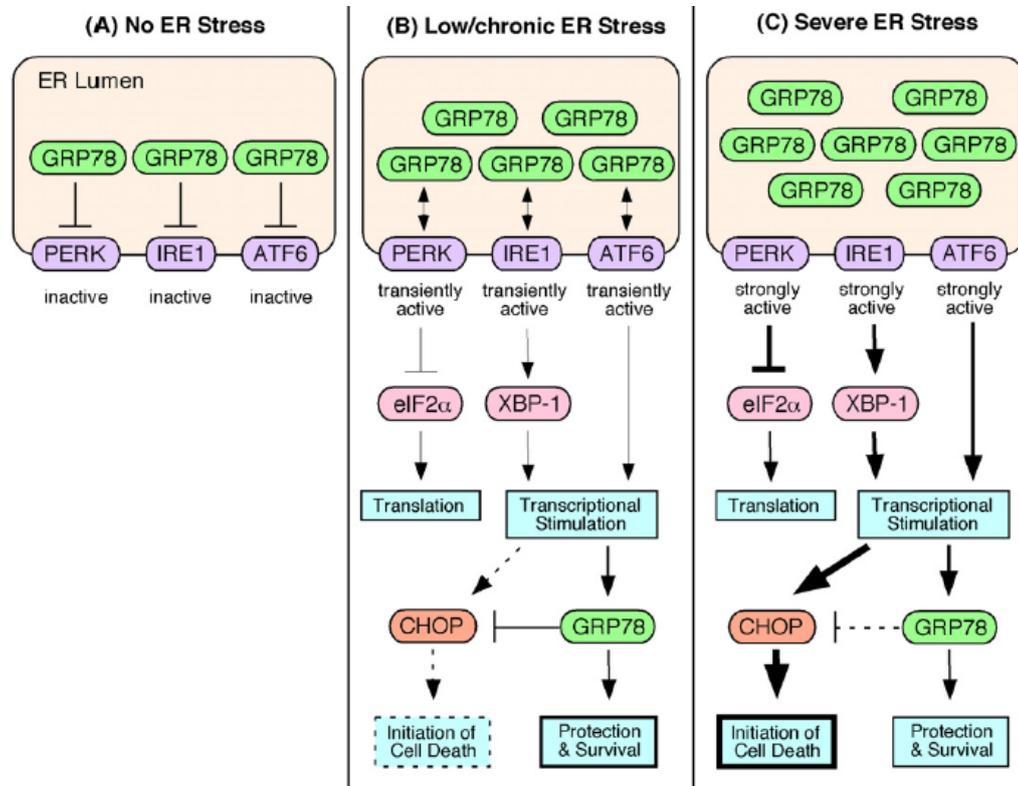
Fig. 2. A. *In a normal cell under physiological conditions, GRP78 binds the ER stress sensors keeping them in a dormant state.* **B**. *In a cancer cell, where there is a chronic level of ER stress, GRP78 dissociates from ER stress sensors, allowing their transient activation. As a consequence, the translation is temporarily reduced and the transcriptional activation of stress genes promotes cell survival.* **C**. *In case of severe ER stress, the apoptotic response, through the activation of CHOP, overwhelms the prosurvival response[44].*
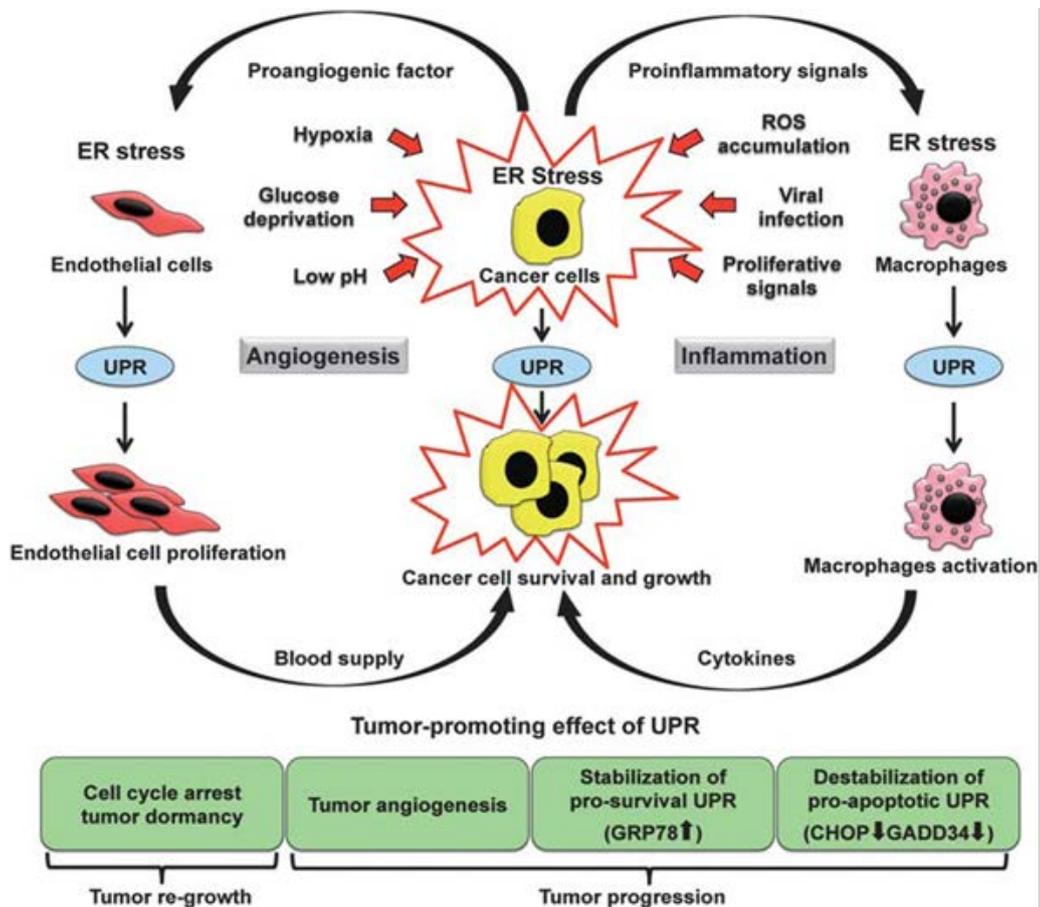
Fig. 3. How ER stress and UPR promote tumor progression. *Cancer cells undergo ER stress due to low oxygen and glucose levels. The adaptive response of the UPR is activated to support the survival and growth of cancer cells. In these conditions cancer cells secrete pro-angiogenic factors to stimulate the proliferation of endothelial cells, which, in turn, promote the survival and growth of the tumor. Cancer cells under ER stress also secrete proinflammatory signals to microenvironment macrophages that, in turn are activated and secrete inflammatory cytokines that further promote tumor growth, angiogenesis, invasion and metastasis.*

1.3.Endometrial cancer

The uterus is the female organ involved in the development of the embryo during pregnancy. It is formed by two main parts: the lower part, called the neck or cervix, in direct connection with the vagina, and the upper part called the body of the uterus, that is formed by different tissues.

In the uterine body, the superficial layer, rich in glands is called the endometrium, while the internal layer, a muscle tissue type, is called myometrium.

The hormonal changes that occur during the menstrual cycle significantly affect the structure of the endometrium which, in case of pregnancy, thickens and feeds the embryo, while if pregnancy does not occur, it degrades and is expelled through the vagina, in the form of menstrual flow.

Endometrial cancer is the most common malignancy of the female genital tract accounting for 6% of all cancers.

The main risk factor for endometrial cancer is age. However, risk factors include obesity, diabetes, estrogen therapy, polycystic ovarian syndrome and westernization of life style[54].

Endometrial carcinomas are classified into Type 1 and Type 2.

Type 1 endometrial carcinomas are typically estrogen-receptor positive, occur in younger women in premenopause and are well differentiated from a histological point of view.

In contrast, type 2 endometrial carcinomas are estrogen receptor negative, more common in older women after menopause, moderately to poorly differentiated and have poor prognosis[55]. The most common form of endometrial carcinoma is type 1 (endometrioid subtype)

According to the classification system FIGO (International Federation of Gynecology and Obstetrics), endometrial carcinoma is graded as low grade (well differentiated) or high grade (poorly differentiated), based on histopathology and evaluation of tumor cell morphology.

In particular:

G1 well differentiated;

G2 moderately differentiated;

G3 poorly differentiated

Additionally, it can be divided into four stages depending on body spreading:

Stage I: The tumor is limited to the uterus body and does not invade the cervix, lymph nodes or other distant sites.

Stage II: Cancer has spread to the cervix, but does not affect areas outside of the uterus.

Stage III: Cancer has spread beyond the uterus, but is still limited to the pelvic region.

Stage IV: Cancer has spread to the bladder, rectum, pelvic lymph nodes and other distant areas as bones or lungs.

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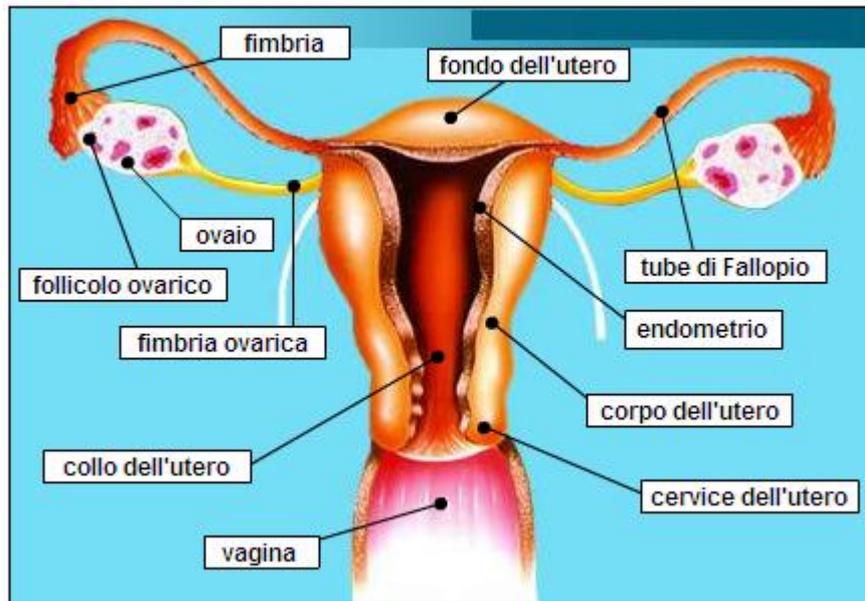
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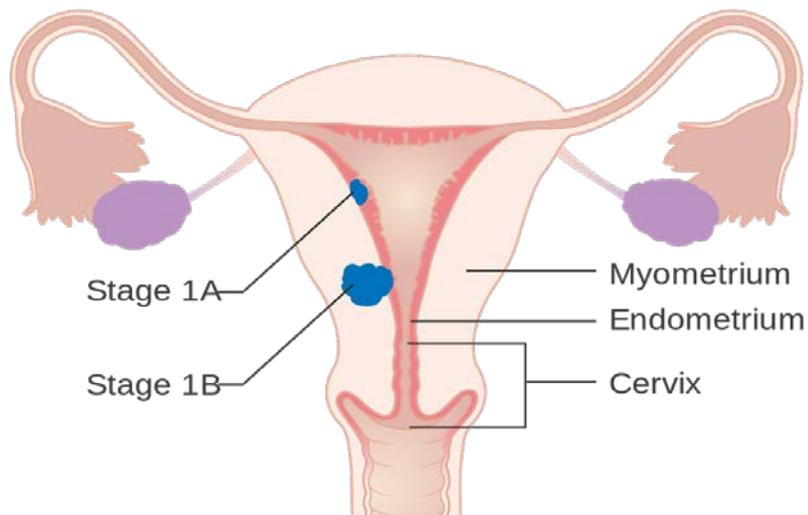
Fig. 4. A. *Female genital.* **B.** *Endometrial cancer.*

1.4.Targeting ER stress for cancer therapy

Chronic ER stress and permanently increased levels of GRP78 expression provide a survival advantage to tumor cells. However, it may represent also an opportunity for therapeutic intervention. It is conceivable that therapeutic exploitation of chronic ER stress present in tumor cells could be approached from two antagonistic directions, as offered by the yin and yang principle of this stress system and the respective key control factors, GRP78 and CHOP.

One obvious approach would entail the blockage of the system's pro-survival yang function (e.g., GRP78), whereas the complementary tackle would be achieved via enhancement of the pro-apoptotic yin process (e.g., CHOP and other components)[56].

Several naturally occurring compounds were found to block GRP78 transcription or inhibit the activity of the protein, such as, the isoflavone genistein, the polyphenolic green tea component (−)-epigallocatechin-3-gallate (EGCG), the microbial metabolites versipelostatin, prunustatin A, efrapeptin J, verrucosidin, deoxyverrucosidin, piericidin A, the plant product arctigenin, the cyanine dye pyrvinium and synthetic members of the biguanide class of compounds (metformin, buformin, phenformin)[56].

However, many of these compounds exert additional, well-recognized biological functions besides inhibition of GRP78 [56].

On the other side, several compounds have been described to aggravate the ER stress response of tumor cells via enhancement of key regulators of the pro-apoptotic arm of the UPR.

Among them, proteasome inhibitors that aggravate the accumulation of superfluous and misfolded proteins, thus leading to chemosensitization (bortezomib); antiretroviral protease inhibitors that have been in use for the treatment of HIV infections that with their protease-inhibitory function can target the enzymatically active core of the proteasome (nelfinavir); COX-2 inhibitors that can inhibit also SERCA proteins thus aggravating ER stress (celecoxib and derivatives); inhibitors of autophagy such as the classic antimalarial compound chloroquine, due to the reciprocal interactions between autophagy and ER stress, where blockage of autophagy leads to aggravation of ER stress.

1.5. Metformin

Metformin is one of the most widely prescribed oral anti-diabetic medications. It is the first line therapy for type 2 diabetes mellitus[57]. It has an anti-hyperglycemic effect which is mediated by inhibiting gluconeogenesis, decreasing glucose absorption from the small intestine, increasing glucose uptake in cells, and decreasing plasma free fatty acid concentration[58]. Metformin also increases insulin induced translocation of glucose transporters to the cellular plasma membrane, thus reducing insulin resistance[59].

Use of metformin has been found to be generally safe, with mild gastrointestinal symptoms being the most common adverse.

Epidemiological studies have demonstrated a correlation between T2DM and a higher incidence of malignancies, especially cancers of the liver, pancreas and endometrium, with approximately a twofold increased risk, as well as cancer of the colon, kidney, bladder and breast, with smaller associations (about 1.2–1.5 fold)[61].

Since epidemiologic data have highlighted the positive effects of metformin to reduce cancer incidence and mortality, many in vitro and in vivo studies, as well as a large number of clinical trials, have been conducted in order to study its potential.

The many anticancer actions of metformin lead to a cytostatic effect. Two distinct but not exclusive mechanisms can be implicated in these actions[62].

First, by decreasing insulinemia and glycaemia, metformin can block the PI3K/MAPK signaling pathway implicated in cell growth.

Second, metformin can directly act on cancer cells by targeting various pathways including tumor metabolism, inflammation, angiogenesis or cancer stem cells, mainly through the activation of the AMPK pathway.

However, an increasing number of studies support the hypothesis that antineoplastic action of metformin can be exerted also through different and AMPK-independent mechanisms.

It has been described, indeed, that metformin decreases the expression of the oncoprotein HER2 (*erbB-2*) in human breast cancer cells via a direct inhibition of p70S6K1 (p70 S6 kinase 1) activity[63].

Furthermore, metformin has been described to suppress epithelial to mesenchymal transition (EMT), a key developmental program that is often activated during cancer invasion and metastasis[64].

Very recently it has been suggested that metformin can inhibit the UPR. Using gene expression profiling techniques, it has been reported indeed that metformin is able to inhibit activators of the UPR and lead to massive cell death in glucose-deprived cultures of human colon, fibrosarcoma, renal, and stomach cancer[65].
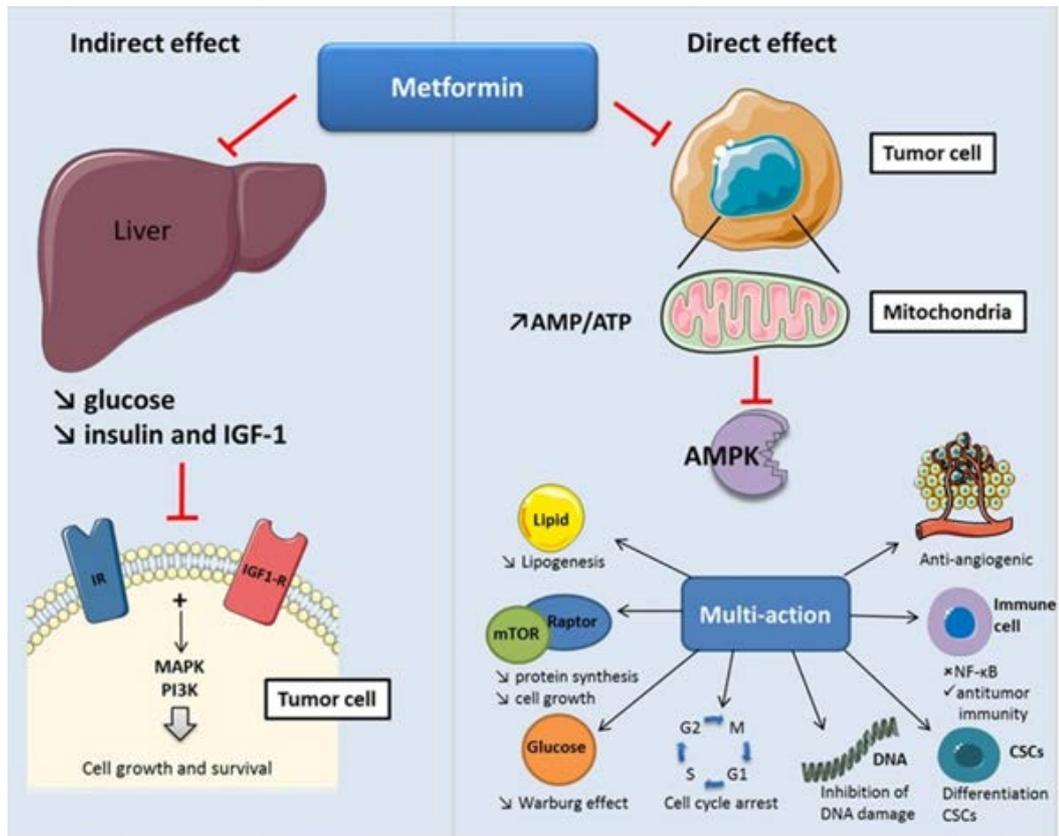
Fig. 5. Focus on metformin antitumour activities. *Accumulating evidence from in vitro and in vivo studies supports the fact that anticancer effects of metformin can be divided into two non-exclusive categories: an indirect effect by reducing the blood glucose and insulin levels, and a direct effect on cancer cells, partially through the activation of AMPK[62] .*

2. AIM OF THE STUDY

Endometrial cancer is the most common malignancy of the female genital tract. However, in spite of a huge advance in our understanding of endometrial cancer biology, therapeutic modalities haven't significantly changed over the past 40 years. There is an increasing interest in the characterization of the molecular mechanisms involved in this pathology, to identify new molecules that could be used, also in association with currently used therapeutic agents, for the treatment of the disease. The aim of this study was to analyze the role of ER stress and the modulation of the UPR in endometrial cancer.

Furthermore, since multiple lines of evidence suggest that metformin exerts anti-tumorigenic effects in different types of cancer, including endometrial cancer, we sought to investigate whether metformin could exert its anticancer activity on endometrial cell through the modulation of the UPR.

The results achieved by this project may represent a good starting point to develop novel therapies for the treatment of this tumor.

3. MATERIALS AND METHODS

3.1. Patients and tissues

Endometrial cancer tissue biopsies (N=12) were collected from women undergoing total abdominal hysterectomy for endometrioid endometrial carcinoma at the Department of Obstetrics and Gynecology of University of Naples "Federico II". All tissues were examined and tumors were graded histologically by a specialist gynecological pathologist according to the guidelines of the International Federation of Gynecology and Obstetrics. 12 patients, matched for age, BMI, and parity, who were undergoing a total abdominal hysterectomy for benign conditions, were used as controls. Vaginal and cervical swabs were performed 2 weeks before the surgical treatment and did not reveal any sign of cervical and/or vaginal infections in any patient. All endometrial specimens collected during surgical procedures were stored at −70 °C. All patients signed an informed consent to participate in the study, which was approved by our Institutional Review Board. The main clinical and anthropometric characteristics of the enrolled patients are reported in Table 1.

3.2. 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, HEC1B (provided by Dr. E. Crescenzi, IEOS-C.N.R.) derived from moderately-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 and HEC1B 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, the anti-GRP78 recognizing the C-terminal domain of the protein, anti ATF6, anti ATF4, anti-CHOP, anti eIF2α, anti-β-actin, anti-p53, anti-PARP, anti-cleaved caspase 9, anti-14-3-3, anti-AKT, anti-p-AKT, anti-ERK1/2, anti-p-ERK1/2, anti AMPK, anti p-AMPK, anti p-S6K, anti β-catenin, anti p-GSK3β and anti PKCα 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).

3.3. 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 mg/ml G418.

3.4. 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 2×10^4 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.

3.5. 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, 1000 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 ml of MTT substrate for 2 h followed by removal of medium and addition of 200 ml 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 b-mercaptoethanol) containing a mixture of phosphatase inhibitors (0.5 mM sodium vanadate, 2 mM sodium pyrophosphate, 5 mM b-glycerolphosphate, and 50 mM sodium fluoride) to prevent postlysis dephosphorylation.

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 (1×10^6 per well) were seeded in six-well plates and allowed to adhere for 24 h. Confluent monolayer cells were scratched by a 200 ml 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.

3.6. Transwell migration assay

Cells (2×10^5) were resuspended in 200 µl of serum-free medium and seeded on the top chamber of the 8 mm 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.

3.7. Immunofluorescence

Tissue sections were deparaffinized and hydrated through xylenes and graded alcohol series followed by antigen retrieval in sodium citrate buffer [0.01 M (pH 6.0)]. Sections were microwaved for 15 min, washed in PBS and PBS containing 0.2% Triton X-100 for 5 min, and incubated for 1 h with blocking buffer.

Tissue sections were incubated o.n. at 4°C with the home made GRP78 anti-serum diluted 1:250 in blocking buffer. AN3CA and Ishikawa cells grown on glass coverslips were fixed with IHC Zinc fixative (BD Pharmingen, San Diego CA) at 4°C for 48 h and then treated without any permeabilization. Nuclei were stained with 5 mM of the DNA intercalator DRAQ5 (Alexis Corporation, Lausen, Switzerland), present in 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 x 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/).

3.8. 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 ml of lysis buffer for 30 min on ice with vortexing every 5 min. The lysates were centrifuged at 10000 x *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 ml of 4x 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/).

3.9.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.

Table 1.
Clinicopathological characteristics of analyzed endometrial carcinoma

Paziente	Età (anni)	BMI (kg/m^2)	FIGO (stadiazione)	FIGO (gradazione)
1	54	36	IA	G1
2	55	37	IA	G2
3	55	42	IA	G2
4	49	23	IA	G1
5	49	30	IA	G1
6	55	45	IA	G1
7	55	33	IA	G2
8	50	40	IA	G1
9	54	35	IA	G2
10	52	28	IA	G1
11	57	34	IA	G1
12	53	33	IA	G2
Controlli (n=12)	52±3	25±3	Nd	nd

4. RESULTS

4.1. mRNA levels of UPR genes are increased in endometrioid endometrial carcinoma

Since it has been described that ER stress is activated in different types of cancer[1,35,38,47], we hypothesized that ER stress might be activated also in endometrial cancer. To test this hypothesis, we collected, 12 tissue samples from endometrial tumors GI stadium and grade G1/G2 and 12 samples of endometrial healthy tissue. The histopathological features of these samples, along with age and body mass index (BMI) of patients, are listed in Table 1.

We analyzed mRNA expression levels of genes activated during the UPR in tissue specimens of endometrioid endometrial carcinoma. As shown by Real-Time RT-PCR experiments, mRNA expression levels of GRP78 and ATF6 were significantly increased in endometrioid endometrial carcinomas when compared to that of normal endometrium (Fig. 6A), suggesting the presence of ER stress and UPR activation in endometrial cancer.

4.2. GRP78 protein expression is increased in endometrioid endometrial carcinoma

To strengthen the Real-Time RT-PCR data, we analyzed GRP78 and ATF6 protein expression in the same tissues. As shown by Western blot experiments (Fig. 6B), also GRP78 and ATF6 protein expression levels were significantly increased, confirming the activation of UPR in endometrioid endometrial carcinomas. Moreover, an immunohistochemical analysis was performed. Tissues of Table 1 were stained with anti-GRP78 antibodies.

A mild staining for GRP78 protein (score 1.3 ± 0.3) was detected in normal endometrial cells, as shown by normal glandular cells of control tissues C2 and C3 (Fig. 7 panels B and C, respectively), whereas glandular and luminal neoplastic epithelial cells from G1–G2 endometrioid endometrial carcinomas K10 and K3 showed a strong (score 3.2 ± 0.6) or a moderate (score 2.3 ± 0.5) increased expression of Grp78 (Fig. 7 panels D and E–F, respectively), correlating with their GRP78 content, previously observed by Western blot experiments. In particular, Grp78 was abundantly present in all epithelial tumor cells with a membrane and a cytoplasmic localization (D–F). Median value for control specimens (n=12) scored 0.9 ± 0.4 ($p<0.05$) whereas that for endometrioid endometrial carcinomas (n=12) scored 2.7 ± 0.6 ($p<0.05$)
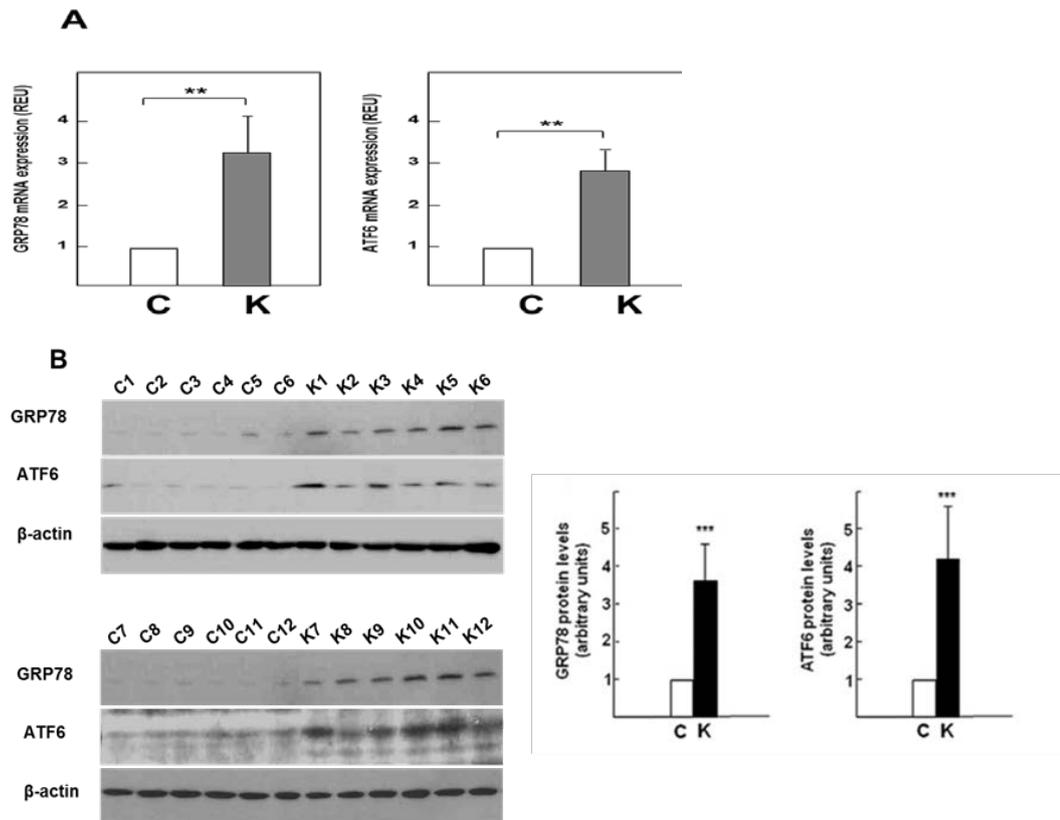
Fig. 6. ER stress markers are upregulated in endometrial adenocarcinoma. A.
*Real-Time RT-PCR analysis of total RNA isolated from tissue specimens of control patients (n=12) with benign conditions and patients (n=12) undergoing surgical treatment for endometrial carcinoma. Each bar represents the mean±s.d. of four independent experiments, each performed in triplicate. (REU, relative expression units). Asterisks indicate statistically significant differences (**, P<0.01).***B. *GRP78 protein expression is increased in endometrioid endometrial adenocarcinoma. Western blots of total protein extracts from 12 normal (C) and 12 endometrioid endometrial carcinoma specimens (K). Filters were probed with antibodies against GRP78 (home-made antibodies), ATF6 and β-actin. The graphs show GRP78 (left panel) and ATF6 (right panel) protein expression levels. Values shown represent the mean (±s.d.) of three independent experiments. ***, P<0.001.*
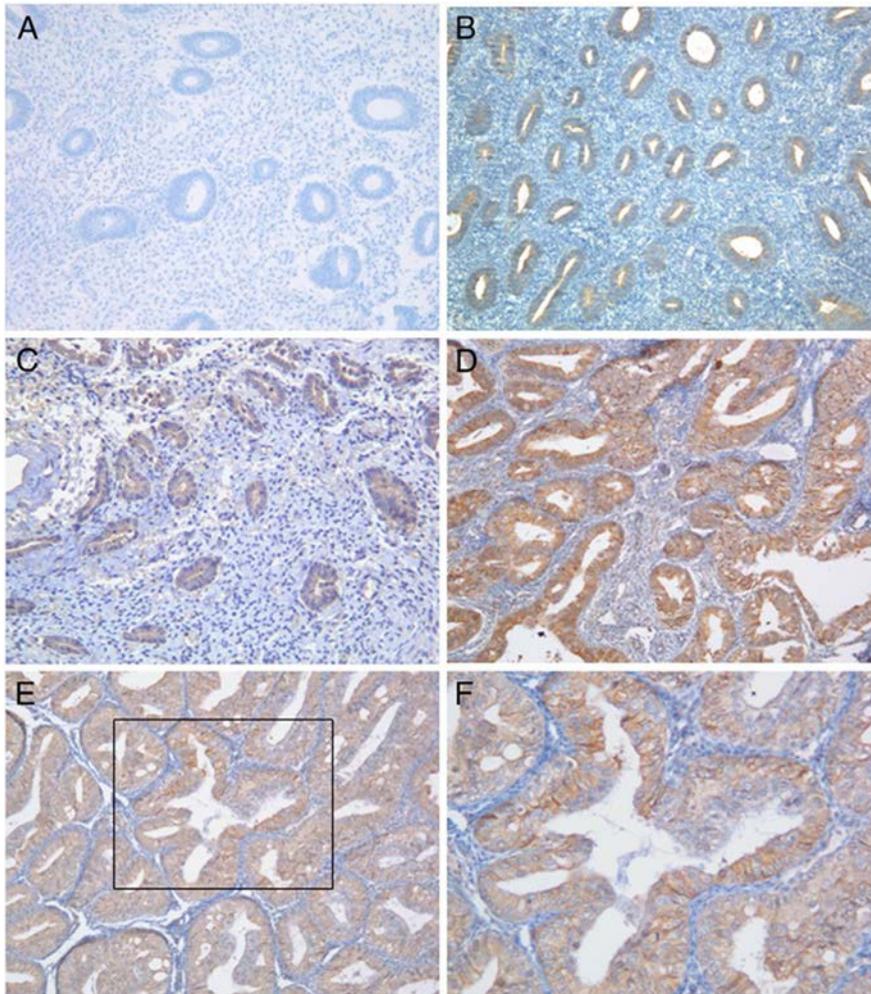
Fig. 7. Immunohistochemical expression for Grp78 in normal endometrium and in Stage I G1–G2 endometrioid endometrial adenocarcinomas. A. *Negative control was obtained using commercial mouse IgG (IHC×106).* **B–C.** *Mild expression of Grp78 in normal endometrial glands (Hematoxylin and eosin×106). C2 (B) and C3 (C) samples are shown.* **D.** *Well-differentiated endometrioid endometrial carcinoma (G1) showing a huge cytoplasmic immunoreactivity (IHC×106). K10 is shown.* **E–F.** *Moderately differentiated endometrial carcinoma (G2) showing a moderate cytoplasmic and membrane positivity of the neoplastic cells (IHC×106). K3 is shown. A high power field is seen in* **F** *(IHC×400). Homemade GRP78 antibodies have been used.*

4.3. 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 8, 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.
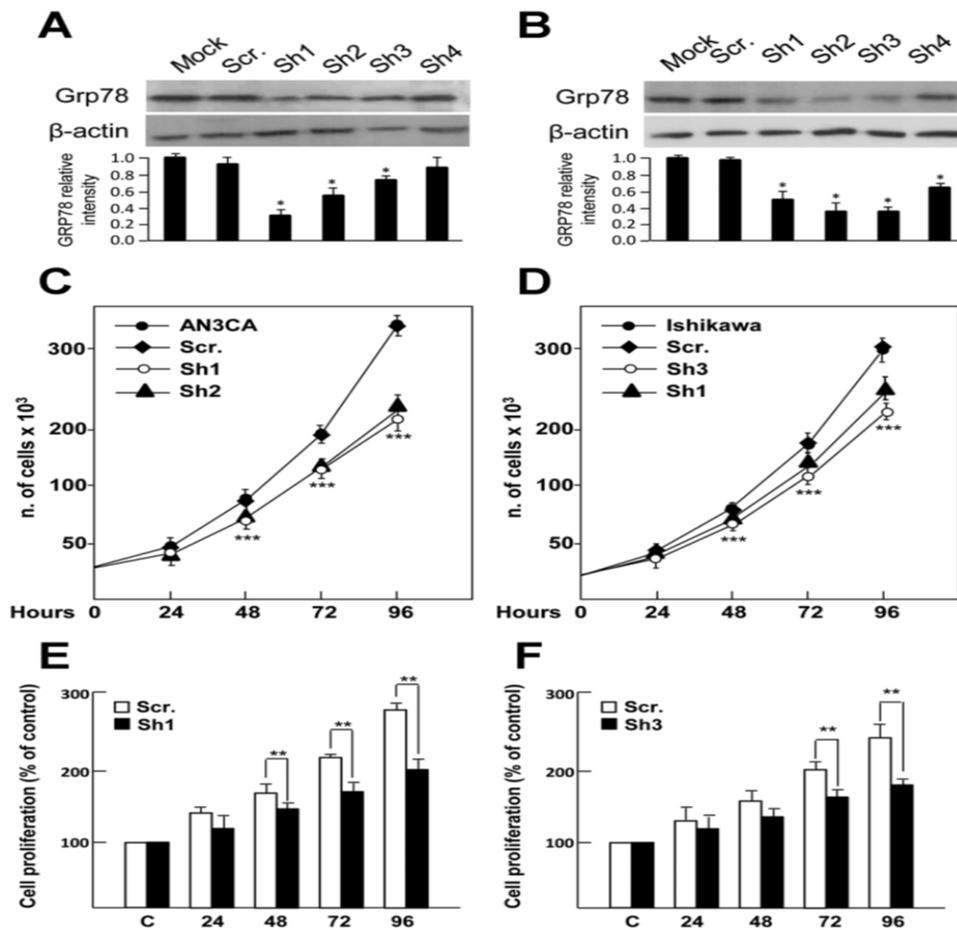
Fig. 8. **The attenuation of GRP78 expression inhibits proliferation of endometrial cancer cells.** *Western blots of total protein extracts from AN3CA (A) or Ishikawa cells (B) untransfected or stably transfected with a construct carrying a scramble sequence (Scr.) or four different sequences specific 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 $2x10^4$ 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 $5x10^3$ 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 540nm ±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.*

4.4. 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 9, 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 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 9 B and C, a significant reduction of invading Sh-transfected cells were found in the lower side of the filter separating upper and lower chamber compared with those Scr-transfected at both time point.

Thus, knockdown of GRP78 expression affected the invasion ability of endometrial cancer cells.
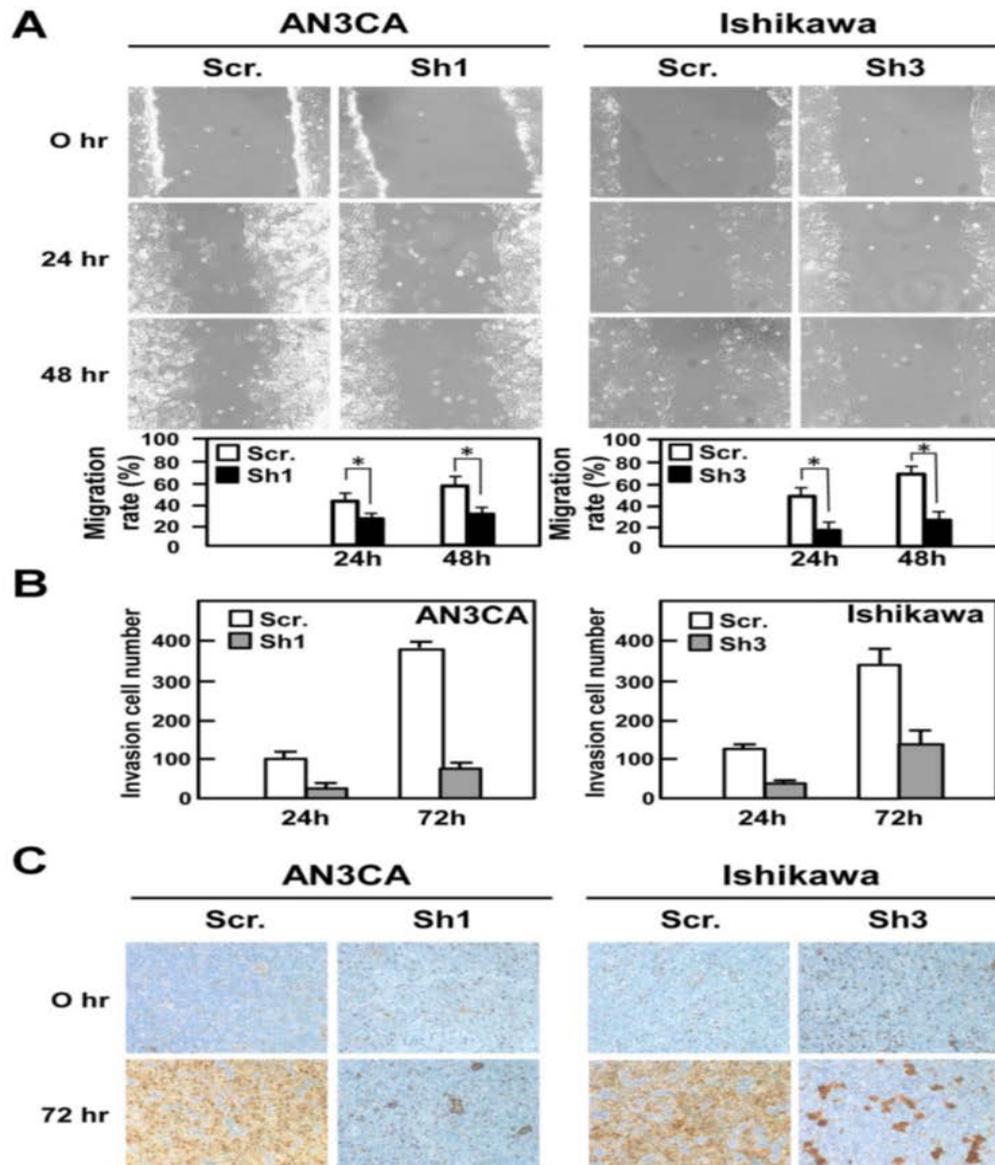
Fig. 9. GRP78 knockdown reduces endometrial cancer cells migration and invasion. A. *AN3CA or Ishikawa cells (1 x 10^6 per well) transfected either with a construct carrying a scramble sequence (Scr.) or a short hairpin sequence specific to human GRP78 (Sh1 or Sh3 respectively), were seeded in six-well 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 x 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.** *Representative experiment of (* **B** *).*

4.5. AN3CA cells express cell surface GRP78

A number of studies have identified GRP78 on the surface of a variety of human cancer cells [43-45,66-74]. 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. 10A).

GRP78 expression and surface localization were significantly increased following tunicamycin treatment (Fig. 10A) in both Ishikawa and AN3CA cells. Consistent with these biochemical findings, confocal microscopy observations confirmed the presence of GRP78 on the cell surface of AN3CA cells (Fig. 10B, part A) but not on that of Ishikawa cells (Fig. 10B, 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. 10B, part B and D, respectively).

A

B
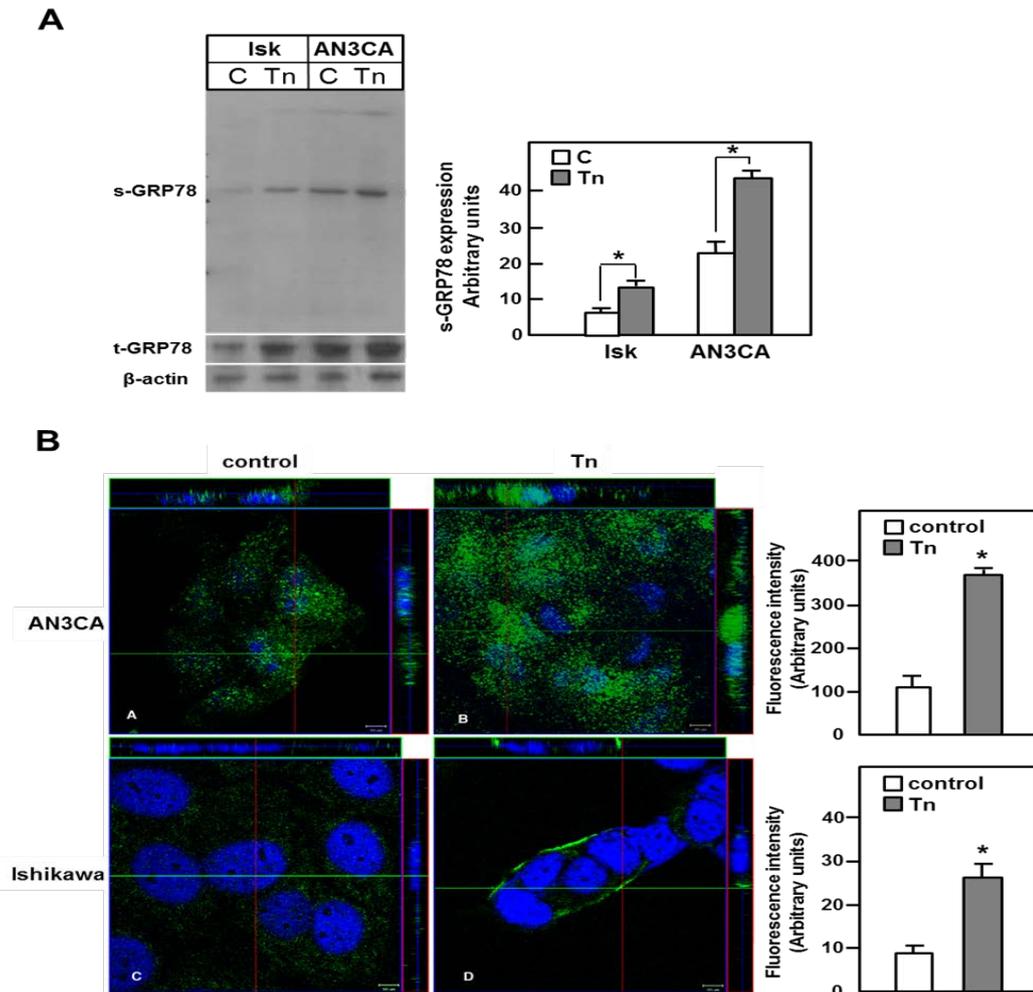
Fig 10 GRP78 localizes on the cell surface of endometrial cancer cells and ER stress further promotes its cell surface localization. **A.** *Detection of cell surface GRP78 expression in Ishikawa and AN3CA cells untreated (C) or treated (Tn) with 1 mg/ml tunicamycin for 24 h. Cells were biotinylated and labeled 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.*

4.6. 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[67-74].

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 11A, following 48 hours 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.11A). To confirm this observation, we performed MTT assays to analyze cell viability of treated versus untreated cells after 24, 48 and 72 hours. As shown in Figure 8B, AN3CA cells treated with the antibodies (+Ab) displayed a significant decline in the percentage of proliferating cells when compared to untreated cells (Fig. 11B). At variance, Ishikawa cells did not show a significant difference in the proliferation rate of treated vs untreated cells (Fig.11C).

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 hours with the antibodies specific to the carboxy-terminal domain of GRP78. As shown in Figure 8D, 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.
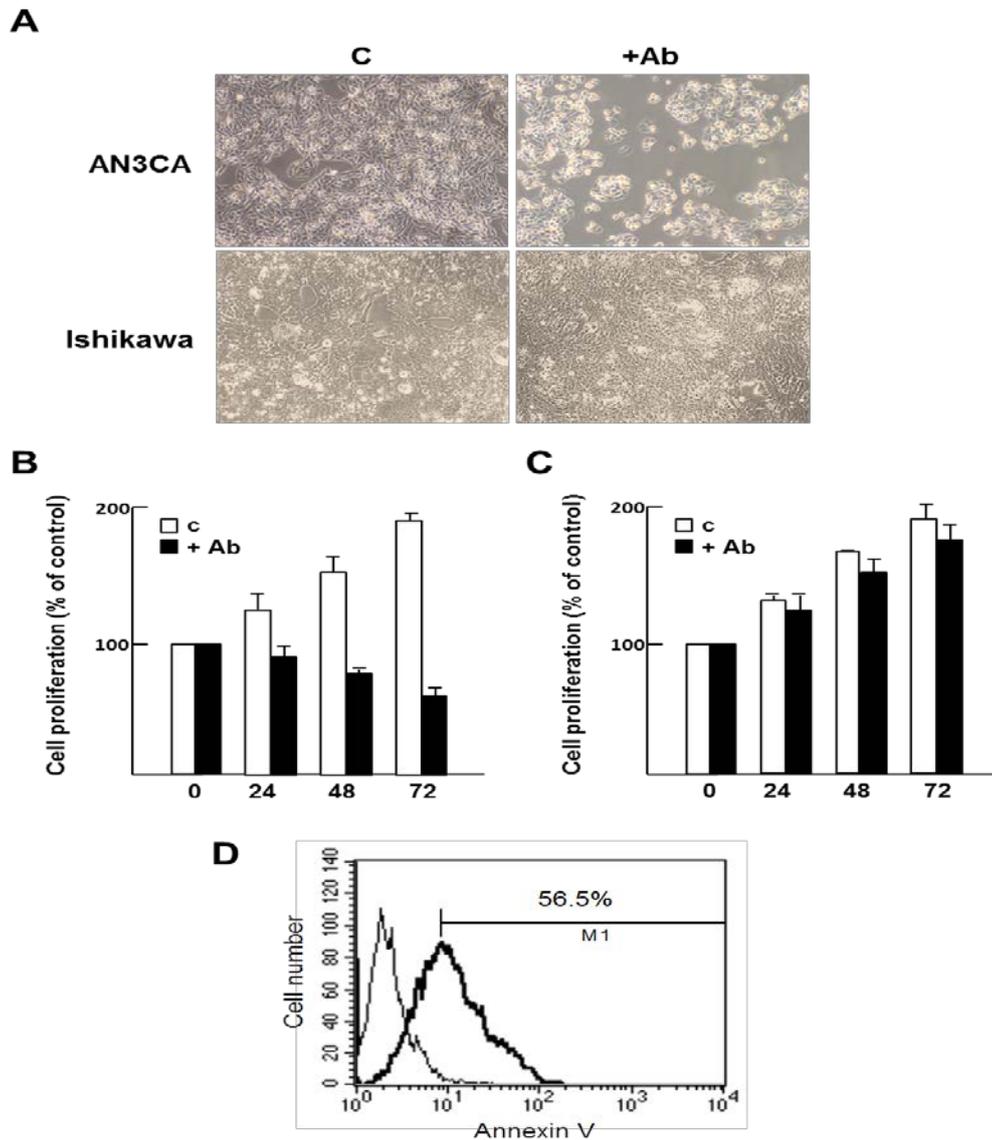
Fig. 11 An antibody recognizing the C-terminal domain of GRP78 induces apoptosis of AN3CA but not Ishikawa endometrial cancer cells. A. *AN3CA and Ishikawa cells were treated or not for 48 hours with 200 µg/ml of an antibody recognizing the C-terminal domain of GRP78. Cells were observed by light microscopy and photographed. AN3CA (**B**) or Ishikawa (**C**) cells were seeded at a density of 5x10³ cells per well in a 96 wells 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 hours 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 hours and subjected to annexin V staining as outlined in Materials and Methods section.*

4.7. 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. 12).

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[70], we evaluated p53 levels in both AN3CA and Ishikawa cells following antibodies treatment.

As shown in Figure 12, 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 12, 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. 12). These data suggest that GRP78 localization on the surface of endometrial cancer cells plays a role in endometrial cancer cells survival and that this might be mediated through the AKT prosurvival signaling pathway.
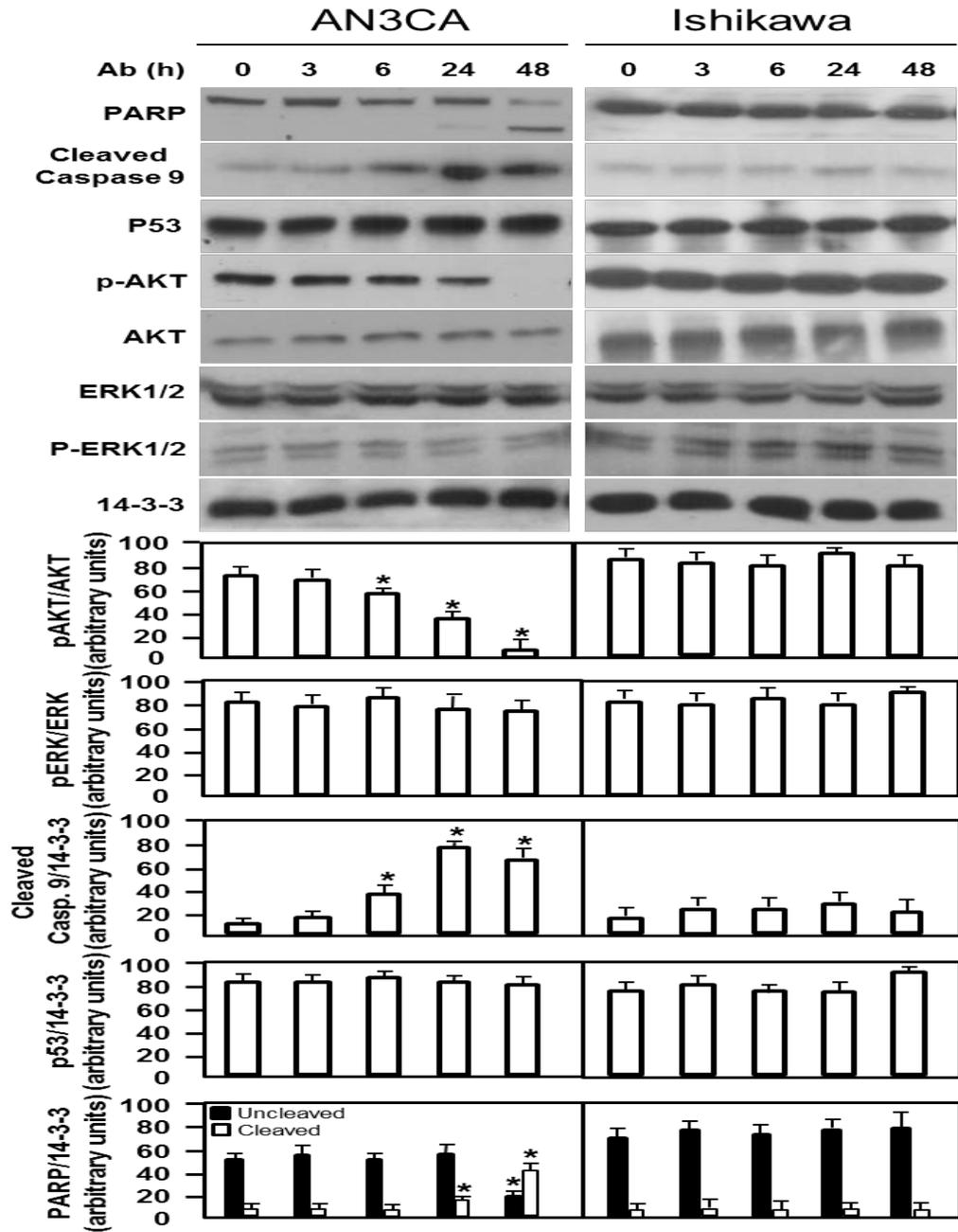
Fig. 12 The treatment with antibodies recognizing the C-terminal domain of GRP78 reduces AKT prosurvival signaling in AN3CA but not in Ishikawa endometrial cancer cells. *AN3CA and Ishikawa cells were left untreated or treated for the indicated times with 200 μg/ ml of an antibody recognizing the C-terminal 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.*

4.8. GRP78 membrane localization is increased in the membrane of endometrial cancer tissues

We have reported that GRP78 is overexpressed in tissue specimens of endometrioid endometrial carcinomas. 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 13, 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.
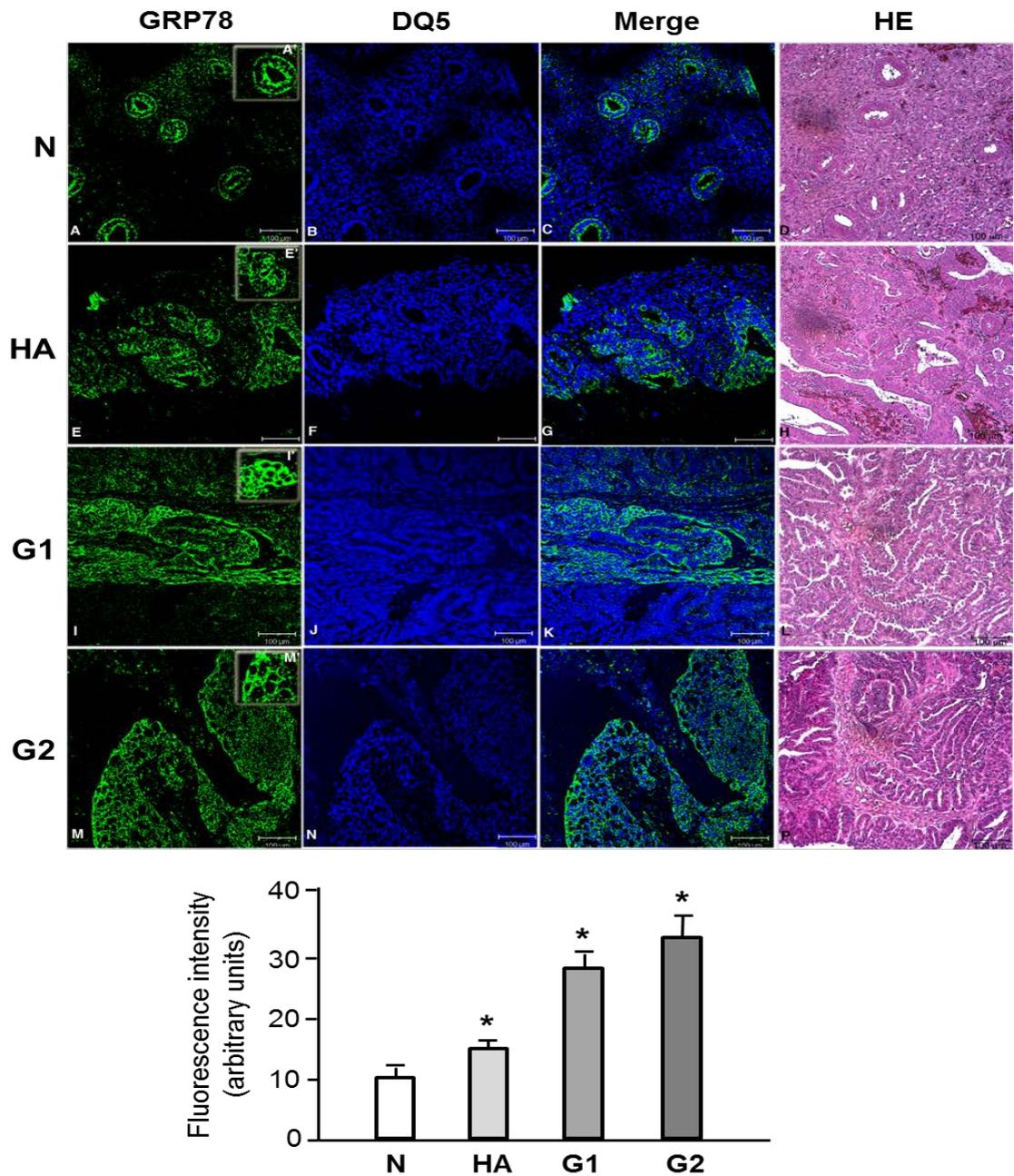
Fig. 13 GRP78 localizes on the membrane of cells of pathological endometrial tissues. *Confocal microscopy images of human endometrial tissue sections representative of normal endometrium (A–D), dysplastic endometrium with atypia (E–H), G1 (I–L), and G2 (M–P) endometrial tumors were immunostained with 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 100µm. Fluorescence intensity measurements of Grp78 staining is also shown.*

4.9. Metformin can reduce vitality in endometrial cancer cells

Since multiple lines of evidence suggest that metformin exert anti-tumorigenic effects in different types of cancer, including endometrial cancer, first of all we evaluated whether metformin could affect endometrial cancer cells growth.

To this aim, we performed MTT assays on Ishikawa, HEC1B and AN3CA cells treated with different concentrations of metformin.

As shown in Figure 15 Metformin inhibits growth in a dose dependent manner in all cell lines. The antitumor activity of metformin has been hypothesized to be due also through a AMPK-independent pathway. We used Compound C (CC), a specific inhibitor of AMPK, in combination with metformin, to evaluate the extent of the AMPK-independent effect of metformin on the vitality of endometrial cancer cells. As shown in Figure 15, the presence of CC did not affect significantly the effect of metformin, suggesting the presence also of AMPK-independent mechanism/s.
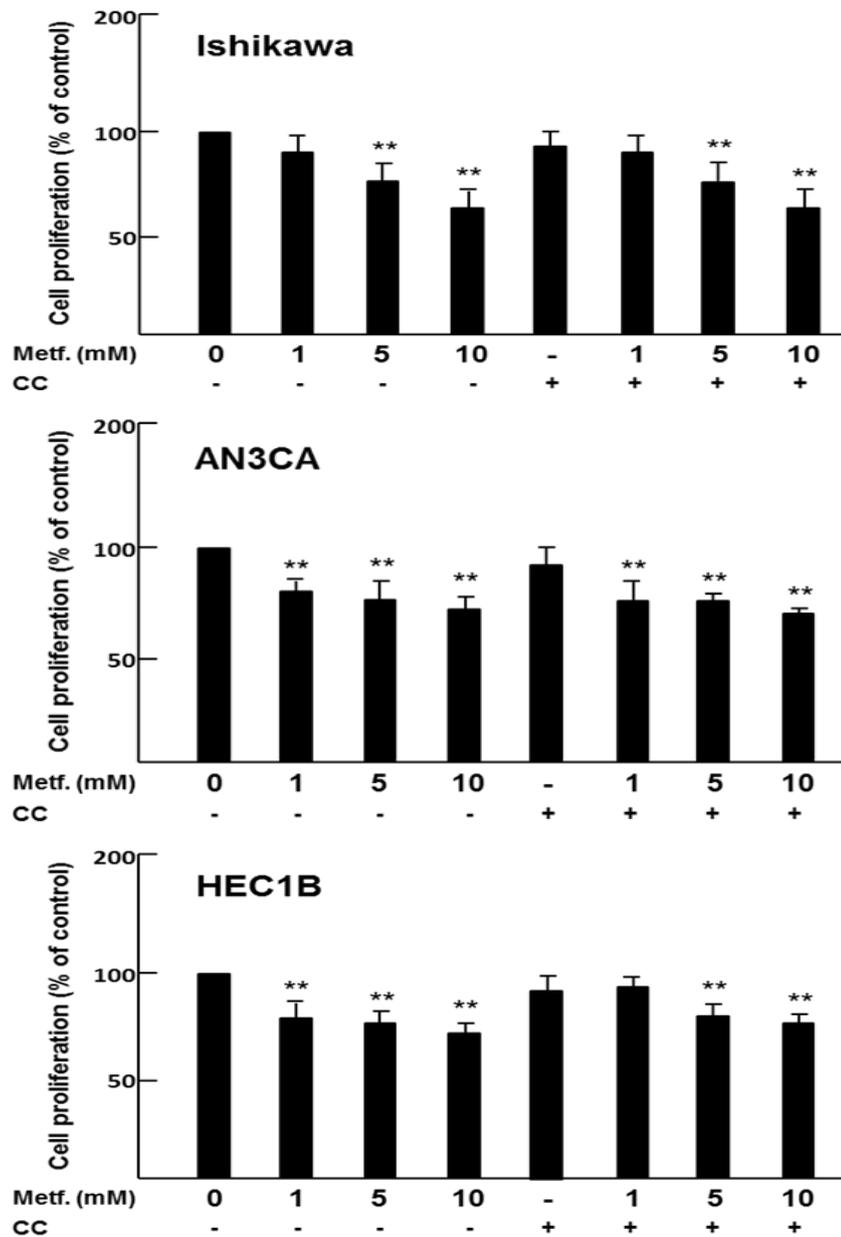
Fig. 14 Metformin reduces vitality of endometrial cancer cells. *Ishikawa, HEC1B and AN3CA cells were left untreated or treated for 24 h with 1, 5 or 10 mM metformin in the absence or presence of 100 nM CC and cell growth was evaluated by MTT assay. The graph represent the median of three different experiments performed in triplicate. **p<0.01.*

4.10. Metformin activates the AMPK pathway in endometrial cancer cells

Metformin induces activation (phosphorylation) of AMPK. Once activated, AMPK induces the inhibition of the mTOR signaling pathway, which results in a lower activation of S6 kinase. Consequently, there is a reduced activation of the ribosomal S6 component and, thus, a decrease of protein synthesis. It has been described that metformin activates AMPK in different cancer cell lines and that this might be the main mechanism through which metformin exerts its cytostatic properties[62].

Metformin was effective in activating AMPK in all endometrial cancer cell lines, as demonstrated by the increase of p-AMPK levels and decrease of p-S6K levels (Fig. 15). furthermore, CC effectively prevented AMPK activation by metformin in all endometrial cancer cell lines.

	Ishikawa				HEC1B				AN3CA			
AMPK												
p-AMPK												
p-S6K												
β-actin												
Met.	-	-	+	+	-	-	+	+	-	-	+	+
CC	-	+	-	+	-	+	-	+	-	+	-	+
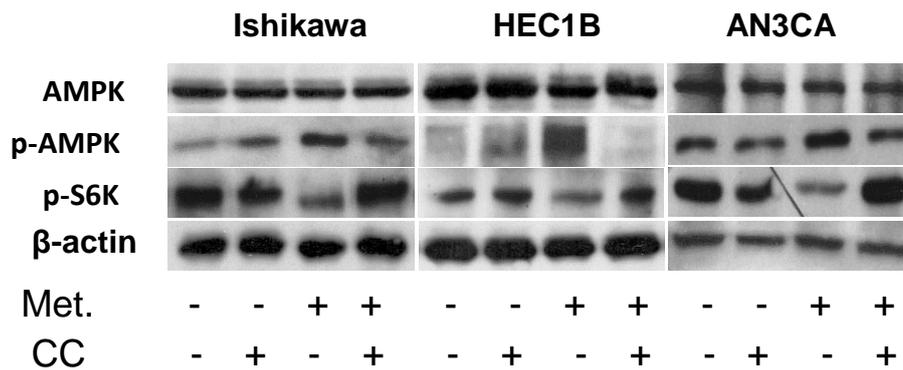
Fig. 15. Metformin induces AMPK pathway activation in endometrial cancer cells. *Ishikawa, HEC1B and AN3CA cells were left untreated or treated for 24 h with metformin in presence or absence of Compound C. Cells were harvested, lysed as described in the "Materials and Methods" section and 50 μg of whole-cell lysates were immunoblotted using anti-AMPK, anti-p-AMPK, anti–p-S6K and β-actin antibodies.*

4.11. Metformin affects mRNA levels of UPR genes in endometrial cancer cells

Very recently it has been hypothesized that metformin might affect the UPR[65].

Thus, we sought to verify whether metformin could modulate the UPR in endometrial cancer cells.

To test this hypothesis, we treated Ishikawa, AN3CA and HEC1B with metformin and we analyzed mRNA expression levels of UPR key genes, such as GRP78, ATF4, ATF6, sXBP1 and CHOP. As demonstrated by the experiments of Real-Time RT-PCR in Figure 16A, the mRNA levels of anti-apoptotic UPR genes (GRP78, ATF6, XBP1) were decreased, while those of proapoptotic UPR genes (ATF4, CHOP) were significantly increased in treated samples when compared to untreated samples, suggesting that metformin modulates the UPR in endometrial cancer cells.

Furthermore, CC was unable to prevent the effect of metformin on UPR genes in endometrial cancer cells, suggesting that the effects of metformin on UPR modulation were at least in part independent from AMPK activation (Fig. 16A).

4.12. Metformin affects the expression of UPR proteins in endometrial cancer cells

To verify whether UPR protein expression levels paralleled those of mRNAs, we performed Western blot experiments on protein extracts obtained from cells treated as above. As shown in Figure 16B, protein expression levels of GRP78, ATF6, XBP1 were significantly decreased while ATF4 and CHOP were increased in the samples treated with metformin, similarly to what observed in Real Time RT-PCR experiments. Again, treatment with CC did not prevent the effect of metformin on UPR proteins expression.
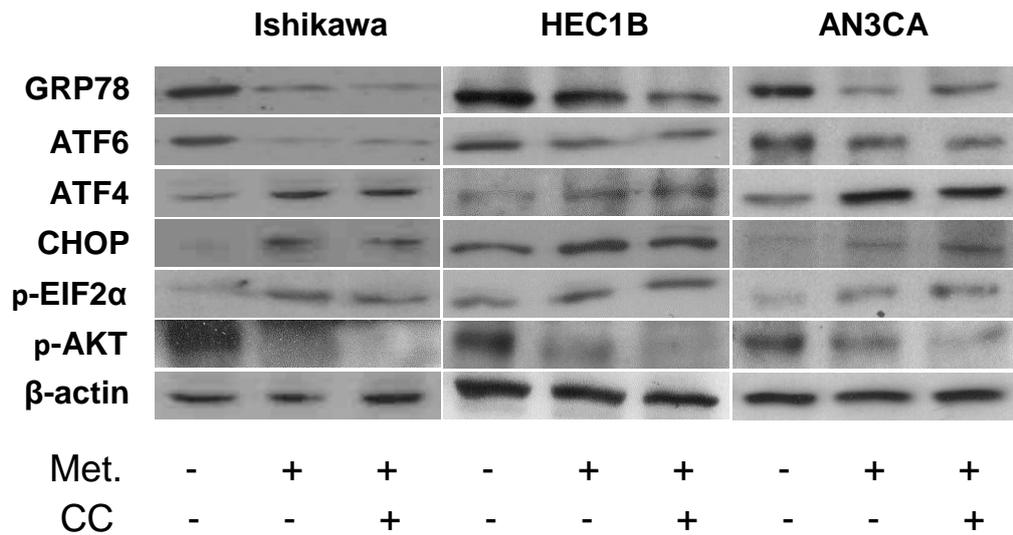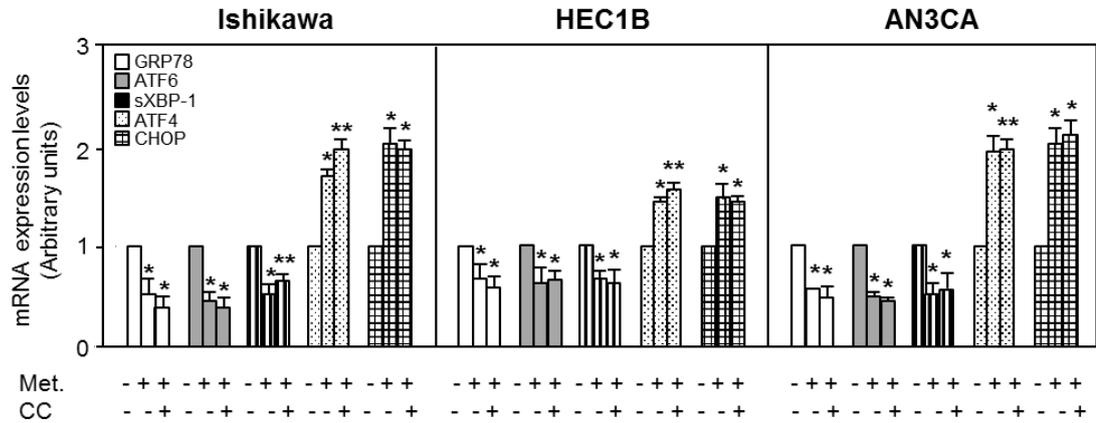
Fig. 16. Metformin affect UPR genes in endometrial cancer. A *Relative mRNA levels of UPR genes (GRP78, ATF6, XBP1, ATF4, CHOP) in Ishikawa, HEC1B and AN3CA cells following metformin and Compound C treatment.* **B.** *Representive Western blots of UPR genes (GRP78, ATF6, ATF4, CHOP, p-eIF2 α) and p-AKT in Ishikawa, HEC1B and AN3CA cells following metformin and Compound C treatment.*

4.13. Metformin downregulates β-catenin levels in endometrial cancer cells

β-catenin is an important protein for the acquisition of several cancer hallmarks by tumor cells. Since GRP78 has been desribed to influence β-catenin protein stability[75] and, thus, β-catenin signaling, we sought to verify whether the observed decrease of GRP78 expression following metformin treatment could affect β-catenin expression in endometrial cancer cells.

As shown in Figure 17, metformin was, indeed, able to decrease β-catenin expression levels in all cell lines. β-catenin downregulation was associated to a decrease of the inhibitory phosphorylation site of GSK3β, thus implicating an increase of the phosphorylation activity of GSK3β on its substrate β-catenin and its increased degradation (Fig. 17). Intriguingly, we observed also activation of PKCα by the metformin (Fig. 17). PKCα has been described to phosphorylate N-terminal serine residues of β-catenin, promoting β-catenin degradation[76].

Immunofluorescence experiments shown in Figure 18 apparently confirmed this observation in that metformin induced the translocation and, thus, activation of PKCα, in the plasma membrane and concomitant downregulation of β-catenin in the same compartment. These results suggest that GRP78 inhibition by metformin could, in turn, affect also β-catenin expression.
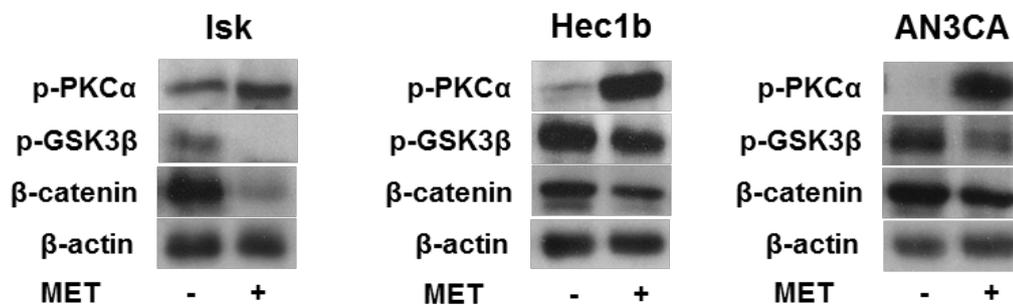
Fig. 17 Metformin downregulates β-catenin levels in endometrial cancer cells. *Western blots of p-PKCα, p-GSK3β and β-catenin in Ishikawa, HEC1B and AN3CA cells untreated or treated with 5 mM metformin.*
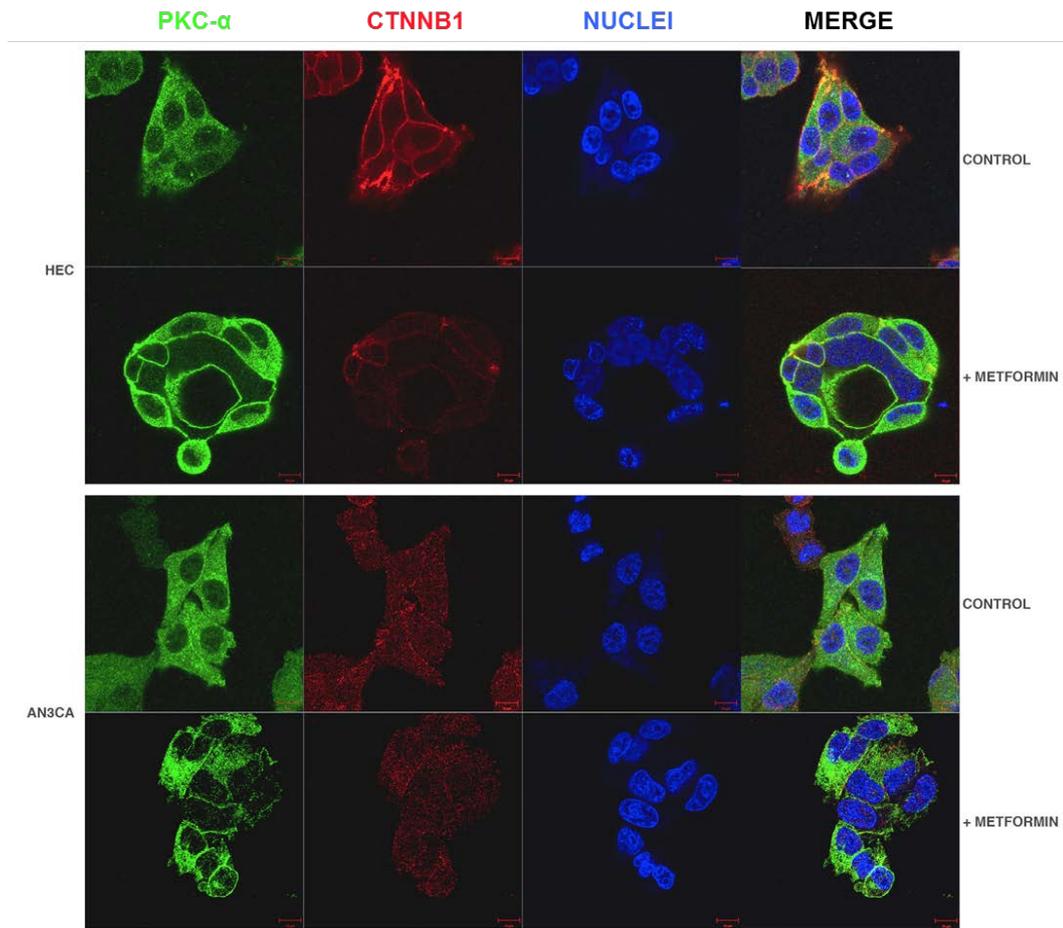
Fig. 18 Metformin modulates β-catenin and PKCα levels in endometrial cancer cells. *Confocal microscopy images of endometrial cancer cells HEC1B and AN3CA. HEC1B andAN3CA cells were not treated (control) or treated with 5 mM metformin. Cells were fixed and stained by indirect immunofluorescence as described in the "Materials and Methods" section using polyclonal β-catenin (red) and PKCα (green) antiserum. Nuclei (in blue) are counterstained with the DNA intercalator DRAQ5. Scale bar 20μm.*

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5. DISCUSSION

Endometrial cancer has become the most frequent gynecologic malignancy in the Western world[77-79]. Although this type of cancer is diagnosed at an early stage in most cases, almost 20% of patients die of their disease[79]. Molecular genetic studies have provided considerable insights into the pathogenesis of endometrial carcinoma. Nevertheless, sufficient prognostic markers have yet to be established.

Recent studies have shown that UPR signaling pathway activation following ER stress plays an important role in tumorigenesis[80]. Following initiation of malignancy, poor vascularization of the tumor mass leads, indeed, to stressful conditions in the tumor microenvironment, including low oxygen supply, nutrient deprivation and pH changes.

These conditions activate a range of cellular stress response pathways, including the UPR[80], and many ER chaperones and UPR target genes show increased expression in human tumor samples.

The relevance of UPR pathways to the survival strategy of tumors is highlighted by the observation that inhibition of UPR adaptive survival responses has been shown to induce selective apoptosis in cancer cells and thus may be highly efficient for cancer treatment. Tumors that lack PERK activity, or compromised in PERK–eIF2α–ATF4 pathways, are small and compromised in their ability to translate mRNAs involved in angiogenesis and tumor survival[81-83]. Disabling the Ire1α-XBP-1 arm of the UPR increases the sensitivity of tumor cells to hypoxic environment and enhances the apoptotic response.

Recently, an increasing number of studies indicate that, among the proteins upregulated following UPR activation, the ER chaperone GRP78, in particular, is critical for tumor proliferation, survival, metastases and resistance to a wide variety of therapies. In a recent study, we reported for the first time that expression of ER stress markers is increased also in tissue specimens of endometrioid endometrial carcinomas, the most common type of endometrial carcinoma, at both mRNA and protein level as shown by ATF6 and GRP78.

In this study, we sought to verify the hypothesis that ER stress and the ER chaperone GRP78 in particular, might have a role in endometrial cancer cells proliferation and/or invasiveness.

We show, indeed, that the inhibition of GRP78 expression by the use of specific sh-RNAs significantly decreases the growth of endometrial cancer cells. GRP78 has been implicated as an important player in 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[84,85]. 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[43]. 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[86]. 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[87]. 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[84,88].

Accordingly, 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 ER resident 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[89-91].

There is evidence for low constitutive surface expression of GRP78 on several cell types, including vascular endothelia[92,93].

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[45,94-96].

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[94]. 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[73].

The proposed mechanism is that this antibody leads to the upregulation of p53, inhibition of NF-κB1 and NF-κB2 activation, and suppression of Ras/MAPK and PI3K/AKT signaling[45,94-96].

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[97]. 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[98].

Downregulating GRP78 expression by the use of specific siRNA, was able, indeed, to decrease AKT activity and to sensitize endometrial cancer cells to apoptosis[98]. 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.

Thus, these data suggest that down-regulating the levels of cellular GRP78 and/or targeting cell surface-associated GRP78, could be of major importance in endometrial cancer therapy.

Metformin is a biguanide drug that is widely used as the first line pharmacologic treatment of type 2 diabetes. However, data obtained in both cell lines and animal models suggests that metformin, besides its antidiabetic action, may exert also anti-tumorigenic effects[62].

Moreover, epidemiological evidence suggest that metformin use lowers cancer risk and reduces the rate of cancer deaths among diabetic patients.

Many of both the systemic indirect and direct effects exerted by metformin on cancer cells are thought to be mediated through the activation of the AMP-activated protein kinase (AMPK), a regulator of energy metabolism usually activated in response to cellular stresses that deplete cellular energy levels increasing the AMP/ATP ratio.

AMPK activation would, in turn, inhibit the mammalian target of rapamycin (mTOR), thereby reducing both protein synthesis and proliferation of cancer cells. However, the underlying biological mechanisms of metformin antineoplastic activity are still largely unknown and it is becoming increasingly evident that AMPK activation by itself is insufficient to explain the plethora of metformin effects on different types of cancer cells. Very recently it has been hypothesized that metformin might affect the unfolded protein response (UPR).

It has been recently shown, indeed, through gene expression profiling techniques, that metformin is able to inhibit activators of the UPR, leading to massive cell death in glucose-deprived cultures of human colon, fibrosarcoma, renal, and stomach cancer[65].

Accordingly, metformin has been described to suppress GRP78, a key driver of bortezomib-induced autophagy, to enhance the anti-myeloma benefit of bortezomib[99]. Thus, we hypothesized that metformin could affect endometrial cancer cells growth, possibly through the modulation of the UPR.

We show, indeed, that metformin inhibits both cell growth and migration in endometrial cancer cells. However, the role of AMPK pathway activation does not appear to be essential for metformin action in endometrial cancer cells. The presence of CC, an AMPK inhibitor, is not sufficient, indeed, to prevent metformin action on this cell type.

We rather observed a modulation of the UPR. In particular, GRP78 expression levels, that have been described to protect cancer cells from apoptosis when overexpressed, were downregulated, while expression of proapoptotic UPR genes, such as ATF4 and CHOP, were upregulated. Furthermore, CC was unable to prevent the observed effects on UPR modulation by metformin, suggesting that these were, at least in part, independent from the activation of the AMPK pathway.

Finally, since it has been described that GRP78 might affect β-catenin pathway, by influencing the stability of the protein[75], we started to analyze the effect of metformin on β-catenin.

Normally, the Wnt/β-catenin pathway controls developmental processes and homeostasis, but abnormal activation of this pathway is a frequent event during the development of cancer, including endometrial cancer[75]. The key mechanism in regulation of the Wnt/β-catenin pathway is the amino-terminal phosphorylation of β-catenin, marking it for proteasomal degradation. In the absence of a Wnt signal, β-catenin forms a complex with adenomatous polyposis coli (APC)/Axin and is phosphorylated by a dual-kinase mechanism, which is catalyzed by casein kinase 1 (CK1) and GSK-3β, at Ser/Thr residues (Ser33, Ser37, Thr41 and Ser45)[100]. However, also other protein kinases, such as PKCα, have been described to directly phosphorylate β-catenin Ser/Thr residues in its N-terminal domain, inducing protein degradation[101].

We observed that metformin is able to downregulate β-catenin protein expression levels in all the endometrial cancer cell lines.

Intriguingly, this effect is associated to the activation of PKCα, suggesting that metformin might inhibit the Wnt/β-catenin pathway in endometrial cancer cells. However, we observed that metformin attenuated GSK3β phosphorylation in the Ser 9 residue and this usually lead to an increase of β-catenin degradation rate[75].

Further experiments are currently ongoing in our laboratory to clarify whether GRP78 has a role in mediating the described effects and to understand whether PKCα action on β-catenin could be direct or indirect through the modulation of GSK3β activity.

6. CONCLUSIONS

In sum, our data suggest that ER stress, the Unfolded Protein Response activation and altered GRP78 expression can play an important role in endometrial cancer.

Based on these data, GRP78 might represent a new therapeutic target for the treatment of endometrial cancer.

Interestingly, we describe also that metformin, an antidiabetic drug, inhibits the growth and migration of endometrial cancer cells, possibly through the downregulation of GRP78 and modulation of the UPR in apoptotic direction.

Metformin effects on the UPR were accompanied to the inhibition of the β-catenin signaling pathway, possibly through the activation of PKCα.

The eventual role of GRP78 in these effects are currently object of investigation in our laboratory.

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