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Glucose regulated proteins in cancer: molecular mechanisms and therapeutic potential

Amy S. Lee
Department of Biochemistry and Molecular Biology, University of Southern California (USC) Norris Comprehensive Cancer Center

Preface

The glucose regulated proteins (GRPs) are stress inducible chaperones majorly residing in the endoplasmic reticulum (ER) and the mitochondria. Recent advances reveal that the GRPs serve distinct functions from the related heat shock proteins (HSPs), and they can be actively translocated to other cellular locations and assume novel functions controlling signaling, proliferation, invasion, apoptosis, inflammation and immunity. Mouse models further identified their specific roles in development, tumorigenesis, metastasis and angiogenesis. This Review describes their discovery, regulation and their biological functions in cancer. Promising agents using or targeting the GRPs are being developed, and their efficacy as anti-cancer therapeutics is also discussed.

Introduction

Glucose regulated proteins, GRP78 (also known as BiP and HSPA5), GRP94 (also known as gp96 and HSP90B1), GRP170 (also known as ORP150 and HYOU1) and GRP75 (also known as mortalin and HSPA9) are stress-inducible molecular chaperones belonging to the heat shock protein (HSP) family (Box 1). Unlike the majority of the HSPs, which reside mainly in the cytosol and nucleus, these GRPs are found in the endoplasmic reticulum (ER) and the mitochondria, which are key organelles regulating protein quality control and metabolic balance[1-4]. In their traditional chaperone roles, these GRPs facilitate protein folding and assembly and the export of misfolded proteins for degradation. Coupled with their Ca^{2+} binding functions, they maintain the integrity and homeostasis of the ER and the mitochondria under physiological and pathological conditions.

GRP overexpression is widely reported in cancer cell lines, associating with aggressive growth and invasive properties[5,6] (Supplemental Table 1). During the past decade, exciting discoveries have been made in identifying common and distinctive functions of these GRPs in cancer. In sustaining ER protein folding capacity and maintaining ER stress sensors and ER associated pro-apoptotic machineries in their inactive state, GRP78 regulates the balance between cancer cell viability and apoptosis[7]. GRP94 is essential for the processing of

Corresponding author: Amy S. Lee, Ph.D., Department of Biochemistry and Molecular Biology, USC Norris Comprehensive Cancer Center, 1441 Eastlake Ave., Room 5308, Los Angeles, CA 90089-9176 USA, Tel.: (323) 865-0507; Fax: (323) 865-0094; amylee@ccnt.usc.edu.

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proteins that have been implicated in tumorigenesis, such as insulin-like growth factor 1 (IGF-1), Toll-like receptors (TLRs) and integrins[4]. GRP170, which has ADP-ATP exchange function, is both a co-chaperone for GRP78 and an independent chaperone, and is critical for vascular endothelial growth factor A (VEGFA) processing and maturation[2,8,9]. GRP75 interacts with the tumour suppressor p53, inactivating the capacity of p53 to function as a transcription factor and inducing apoptosis[10]. Furthermore, these GRPs, traditionally regarded to reside exclusively in the ER lumen, can be actively translocated to other cellular locations and secreted, and have additional functions that control signaling, proliferation, invasion, apoptosis, inflammation and immunity[11-14]. ER stress, as well as development of therapeutic resistance, actively promotes cell surface expression of GRP78, which serves as an upstream regulator of the PI3K-AKT oncogenic signaling pathway[15-17]. GRP78 is also a downstream target of AKT activation[18,19]. At the cell surface, GRP94 and GRP170 function in antigen presentation, and their secreted forms have the ability to elicit innate and adaptive immune responses, which could be useful in the development of cancer vaccines[1,2,20].

Through the use of cancer cell lines, xenografts and conditional knockout mouse models, the important roles of these GRPs in cancer are being established[5,20,21]. Promising therapeutics specifically directed against the GRPs, including conjugated peptides and toxins, antibodies, small molecules and microRNAs, are being developed[5,20,22]. Thus, these GRPs represent novel prognostic markers and targets[5], as well as mediators or vaccines for anti-cancer therapy[2,23] that warrant vigorous investigation.

GRPs in the stress response

The GRPs are ubiquitous chaperones that are constitutively expressed at basal level and that sustain organ homeostasis through different mechanisms (Supplemental Table 2). The induction of the GRPs is widely used as an indicator for the onset of ER stress and studies into their transcriptional activation mechanism (Box 2) have facilitated the discovery of novel intracellular signaling pathways whereby stress from the ER can be communicated to the nucleus to initiate transcription of the unfolded protein response (UPR)-associated genes[20,24,25]. Cancer cells are subjected to ER stress triggered by both intrinsic and extrinsic factors, such as altered cell metabolism, hyperproliferation, hypoglycemia, hypoxia, acidosis, viral infection and genetic lesions leading to the production of mutated proteins that misfold[20,26]. These adverse conditions impinge on proper protein folding in the ER, creating ER stress. GRP78 regulates the UPR by binding to and inactivating all three ER stress transducers [PRKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6)][7,20] under non-stressed conditions. When misfolded proteins accumulate in the ER, GRP78 binds to them, thereby releasing the UPR sensors and leading to the activation of the UPR pathways[27-31] (Figure 1). Conversely, when GRP78 is depleted or inactivated, the UPR can be triggered spontaneously, with diverse physiological consequences[32-35] (Supplemental Table 2). Nonetheless, GRP induction is not limited to ER stress. For example, autophagy-defective tumor cells upregulate ER chaperones in response to metabolic stress[36], and histone deacetylase inhibitors activate *GRP78* transcription without concomittantly inducing stress response in general[37] (Box 2).

Biological functions of the GRPs in cancer

As summarized in this section, the GRPs, in both UPR-dependent and UPR-independent functions, have important roles in regulating a variety of processes that are essential for tumorigenesis at multiple cellular locations (Figure 2).

Proliferation

GRP78 expression levels correlated with proliferative rates of human glioma cell lines and knockdown of GRP78 by small interfering RNA (siRNA) suppresses their growth[38]. In a mouse mammary tumour virus (MMTV)-Polyoma middle T (PyT) mammary tumour model, *Grp78* heterozygosity was sufficient to prolong the latency period and impede cancer growth in part through suppression of tumour cell proliferation[39] (Table 1). How might GRP78 facilitate proliferation? As an ER chaperone, GRP78 controls processing and maturation of a wide variety of cell surface receptors and secretory proteins crucial for the ability of cancer cells to respond to extrinsic proliferative signals[20]. GRP78 is also a regulator of Wnt-β-catenin proliferative signaling through its stabilization of Wnt in the ER. When GRP78 was dissociated from Wnt under hypoxic conditions, Wnt was not properly processed, leading to its proteasomal degradation and reduced Wnt secretion[40].

GRP78 might also promote cell proliferation from the cell surface. ER stress or ectopic expression of GRP78 leads to localization of a subfraction of GRP78 on the cell surface[15]. Specific proteins have been reported to transport GRP78 to the cell surface in different cell types, such as the carrier protein MTJ-1 in macrophages and the tumour suppressor prostate apoptosis response 4 (PAR-4; also known as PAWR) in the prostate cancer cell line PC-3[41,42].

Cell surface GRP78 acts as a multifunctional receptor impacting both cell proliferation and viability[11-14]. For example, in prostate cancer cells, cell surface GRP78 serves as receptor for the activated form of the plasma proteinase inhibitor α2-macroglobulin (α2-M*)[43], triggering ERK and AKT activation and increased DNA and protein synthesis[44]. AKT signaling, which promotes proliferation and inhibits apoptosis, is also triggered by autoantibodies against the N-terminus of GRP78 that are found in cancer patients[45]. How might GRP78 regulate AKT activation? Cell surface GRP78 co-localizes with PI3K, an activator of AKT, and co-immunoprecipitates with PI3K subunits[16,17]. Furthermore, in cell culture model systems, overexpression of GRP78 leads to increased PIP3 production (a signaling molecule downstream of PI3K) and mutation of the N-terminal region of GRP78 reduced both the binding of cell surface GRP78 to PI3K and PIP3 production[16]. A requirement for GRP78 in a serum-stimulated increase in PIP3 production has also been reported in human leukemic cells[34].

PTEN (phosphatase and tension homolog deleted in chromosome 10), which encodes a plasma membrane lipid phosphatase that antagonizes the PI3K signaling pathway, is a major tumour suppressor gene in human cancer[46]. A biallelic conditional knockout mouse model of *Grp78* and *Pten* in the prostate epithelium or bone marrow showed that GRP78 deficiency reduces PI3K-AKT activation, which normally occurs as a result of PTEN loss in these cells, and potently inhibits prostate tumorigenesis[47] and leukemogenesis[48],

respectively (Table 1). Although cell surface GRP78 has been shown to regulate PI3K signaling, further studies are required to determine whether GRP78 in the ER or other cellular locations might also regulate PI3K-AKT signaling. Recent studies revealed GRP78 is a downstream target of the IGF-1R-PI3K signaling pathway in mouse embryo fibroblasts, as well as in cancer cell lines[18,19], and this could represent a feedback regulatory mechanism that balances GRP78 expression and cancer cell proliferation.

Another pro-proliferative mechanism of GRP78 is the interaction of cell surface GRP78 with Cripto-1 (also known as teratocarcinoma-derived growth factor 1), a glycosylphosphatidylinositol (GPI)-anchored, developmentally regulated, oncoprotein.[49] Disruption of cell surface GRP78 and Cripto complex blocked Cripto activation of MAPK and PI3K pathways and modulation of activin-A, activin-B, nodal and transforming growth factor-β1 signaling[50]. Thus, cell surface GRP78 is a necessary mediator of Cripto proliferative signaling in human cancer.

GRP94 controls the maturation and secretion of IGFs, which are important mitogenic factors[51], and binding of IGF-1 or IGF-2 to the IGF-1R leads to PI3K-AKT activation. GRP94 regulates the processing of the low density lipoprotein receptor-related protein 6 (LRP6), a Wnt co-receptor[52]. Without GRP94, LRP6 is not exported from the ER to the cell surface, leading to attenuation of the pro-proliferative and pro-survival Wnt-β-catenin signaling pathway. This is the proposed mechanism for the attenuation of multiple myeloma and inflammatory colorectal cancer in mouse models where *Grp94* is deleted in B-cells[53] and macrophages[54], respectively (Table 1). In breast cancer cells that are able to proliferate under chronic exposure to reactive oxygen species (ROS) *in vitro*, the expression of GRP94, but not HSP90 or GRP78, is increased[55]. ROS are counteracted by the production of antioxidants and the formation of disulphide bonds in proteins in the ER, which is promoted by GRP94.

Overexpression of GRP75 in mouse fibroblasts leads to anchorage-independent growth, and formation of tumours when transplanted into nude mice[56]. Contributing factors might include the role of GRP75 as a mitochondrial protein importer and its ability to retain p53 in the cytoplasm, leading to down-regulation of p53 target genes such as *Cdkn1a* and *Mdm2*. This effect on p53 has been shown in a subset of neuroblastomas[57]. Another client protein of GRP75 is fibroblast growth factor-1 (FGF-1) which possesses broad mitogenic activities and functions as a modifier of endothelial cell migration and proliferation and is therefore pro-angiogenic[58].

Apoptosis

In general, the GRPs are suppressors of apoptosis[20]. Caspase-7, an executioner caspase that is associated with the ER, can be activated by the chemotherapeutic agent etoposide, and GRP78, in a manner dependent on its ATP binding activity, forms complex with caspase-7 (Figure 1) and protects cells from apoptosis induced by etoposide[59,60]. Recently, a functional relationship was uncovered at the outer surface of the ER between GRP78, the pro-apoptotic protein BIK and the anti-apoptotic protein BCL-2[61,62]. GRP78 and BCL-2 form separate complexes with different domains of BIK. BIK sequestration of BCL-2 reduces BCL-2 interaction with the ER, leading to ER Ca^{2+} release, translocation of the pro-

apoptotic protein BAX to the mitochondria and the release of cytochrome *c* to the cytosol, which initiates apoptosis. However, high levels of GRP78 sequester BIK, which releases the inhibition of BCL-2, thereby suppressing apoptosis[62].

These observations, however, raise the important question of how GRP78 as an ER lumen protein, can interact with cytosolic proteins that associate with the outer ER membrane. Intriguingly, two independent studies showed that a subpopulation of GRP78 from isolated microsomes was resistant to sodium carbonate extraction and existed as a partially protease resistant (presumably transmembrane) protein[59,60]. However, despite the presence of some weak hydrophobic motifs supporting this possibility, the primary amino sequence of GRP78 does not predict a traditional transmembrane configuration under normal physiological conditions. Thus, the interaction between GRP78 and cytosolic proteins will have to be mediated either by an unconventional form of GRP78 that spans the ER membrane or lumenal GRP78 in complex with other ER transmembrane proteins; this issue remains to be resolved.

ER stress induces alternative splicing of *GRP78*, generating a cytosolic isoform (GRP78va) that regulates PERK signaling and enhances leukemia cell survival[63]. ER stress also promotes GRP78 localization to the mitochondria, which are physically and functionally interconnected with the ER (Box 3). Mitochondria-associated GRP78 can bind to RAF1 and this interaction is involved in the maintenance of mitochondrial permeability and thus protective against ER-stress-induced apoptosis[64]. In support of the anti-apoptotic functions of GRP78, knockout of *Grp78* in various tissues led to caspase activation and tissue atrophy[33,34,65,66] (Supplemental Table 2). In breast, prostate and leukemic cancer models, heterozygous and/or homozygous knockout of *Grp78* increased tumour apoptosis and impeded tumour progression[39,47,48] (Table 1).

Through interaction with α2-M*, cell surface GRP78 promotes 1-LN prostate cancer cell survival by activating the AKT and NF-κB signaling cascades[67]. In hypoxic HT1080 fibrosarcoma cells, cell surface GRP78 serves as a receptor for Kringle 5, a human plasminogen factor, which upon internalization, competes with procaspase-7 binding to the ATP binding domain of ER GRP78, leading to caspase-7 activation and tumor cell apoptosis[68]. However, one study indicates that cell surface GRP78, along with PAR4 has a pro-apoptotic function through mediating TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFSF10) activation, triggering the extrinsic apoptotic pathway in PC3, HeLa and H460 cells[42]. Nonetheless, in MCF-10A and MDA-MB-231 cells, GRP78 prevents TRAIL-induced apoptosis and is therefore a pro-survival factor[69]. Thus, the effect of GRP78 on TRAIL-induced apoptosis may be context dependent. Besides modulating apoptosis, GRP78 has been implicated in protective autophagy through maintenance of ER structural integrity[32], and modulation of mTOR signaling in estrogen resistant breast cancer cells[70].

GRP94 maintains ER Ca^{2+} homeostasis and protects cancer cells from apoptosis[71]. GRP94 deficiency in human multiple myeloma cells resulted in apoptosis through inhibition of the Wnt-survivin pathway[53]. In the SkBr3 breast cancer cell line that abundantly expressed cell surface HER2 (also known as ERBB2), pharmacological inactivation of GRP94 destabilized

HER2 and inhibited RAF1-MAPK survival signaling at the cell membrane[72]. In cancer cells, GRP170 is upregulated by hypoxia and by drugs such as celecoxib and proteasome inhibitors, and knockdown of GRP170 activated the expression of the UPR pro-apoptotic factor CHOP and stimulated apoptosis[73,74]. GRP170 may also protect cancer cells against cell death through blocking ER Ca^{2+} release or delaying the onset of UPR by binding to the ER stress sensors[75,76].

Angiogenesis

Eliminating the tumour vasculature, which supplies nutrients and oxygen within the tumour, is a key strategy for anti-cancer therapy. Tumour-associated endothelial cells are physiologically and functionally different from endothelial cells derived from normal tissues, and express high levels of GRP78 compared with normal organs[38,77,78]. *Grp78*$^{+/-}$ mice, as well as mice with conditional heterozygous knockdown of *Grp78* in the host endothelial cells, showed a dramatic reduction in tumour microvessel density (MVD) while having no effect on the MVD of normal organs[39] (Table 1). *Grp78* knockdown impairs immortalized endothelial cell proliferation, survival and migration *in vitro*, supporting its requirement for neoangiogenesis in primary tumour and metastatic growth[39].

GRP78 is expressed on the surface of proliferating endothelial cells[68,79]. Cell surface GRP78 assoicates with the GPI (glycosylphosphatidylinositol) anchored T-cadherin and mediates T-cadherin-dependent endothelial cell survival[80]. In exploiting cell surface GRP78 for anti-angiogeneis therapy, plasminogen Kringle 5 is reported to bind GRP78 in glioma endothelial cells for induction of apoptosis[68], which can be sensitized by radiation dependent on internalization of cell surface GRP78 by the low-density receptor-related protein and activation of p38 MAK[78]. Other studies however suggest that the apoptotic effect of Kringle 5 in proliferating endothelial cells and 1-LN prostate cancer cells was mediated by the cell surface voltage-dependent anion channel which co-localizes with GRP78 and is regulated by GRP78[81]. VEGF can induce cell surface expression of GRP78 in endothelial cells and knockdown of GRP78 suppressed VEGF-mediated MAPK signaling and endothelial cell proliferation[82]. On the other hand, knockdown of GRP170, while having no effect on GRP78 and GRP94 expression, resulted in retention of VEGF in the ER and blocked its secretion[83]. Collectively, these studies suggest that targeting GRP78 and GRP170 could achieve a dual effect in suppressing tumour growth as well as tumour angiogenesis.

Invasion and metastasis

Tumour metastasis is a multistep process and requires enhancement of specific tumour cell properties including degradation of the extracellular matrix (ECM), migration, invasion, angiogenesis and survival. The level of intracellular GRP78, as well as cell surface GRP78, is increased in metastatic cancer cell lines, lymph node metastases and human metastatic lesions[6,84-86]. Knockdown of GRP78 suppresses tumour cell invasion *in vitro* and suppresses metastatic growth in xenograft and syngeneic tumour models[87-89]. In addition to protecting metastatic tumour cells from the adverse host environment and promoting angiogenesis, GRP78 has been shown to promote cell motility. One mechanism is through cell surface GRP78 acting as co-receptor for ligands signaling the activation of kinases

known to enhance migration, such as AKT, focal adhesion kinase (FAK) and p21-activated kinase 2 (PAK2)[88,90]. It has also been proposed that cell surface GRP78 acts as a bridge protein for the urokinase-type plasminogen activator (uPA-uPAR) protease system, which can mediate degradation of the ECM and promote invasion[88]. Like GRP78, GRP94 overexpression is associated with lymph node metastasis and carcinoma recurrence, and silencing of GRP94 inhibits migration and proliferation of MDA-MB-231 breast cancer cells *in vitro*[55,91]. GRP94 client proteins include cell interaction and cell matrix component, such as integrins, which might explain its influence on cell invasion. Recently, it was demonstrated that a cell-permeable peptide that competitively inhibited the interaction between GRP94 and integrins blocked cell invasion[92]. GRP75 overexpression is associated with liver cancer metastasis[93], and GRP170 upregulation is observed in invasive breast cancer[94] (Supplemental Table 1). Thus, the GRPs are novel protein targets for the inhibition of cancer metastasis.

Inflammation and immunity

ER stress can drive a pro-inflammatory program in tumour cells and macrophages that facilitates tumour progression. Additionally, stressed tumour cells secrete mediators that stimulate macrophages to produce pro-inflammatory cytokines, further amplifying the pro-inflammatory response of tumour cells[20,95]. On the other hand, cancer cell survival requires resistance against host immune defenses. GRP78 regulates inflammation and immunity through multiple mechanisms[20,96]. As a major ER chaperone, GRP78 facilitates the processing and secretion of cytokines and chemokines[96,97]. Acute ablation of *Grp78* in adult mice results in alteration of their chemokine and cytokine profile[34] (Supplemental Table 2). In terms of immune evasion, GRP78 protects fibrosarcoma cells from lysis by cytotoxic T lymphocytes (CTL) and tumour necrosis factor *in vitro*, and when fibrosarcoma cells incapable of inducing GRP78 were injected into mice, tumours were either not formed or rapidly regressed with evidence of cytotoxic T cell response[98].

GRP78 is an obligatory binding partner for cell surface major histocompatibility complex (MHC) class I molecules[99]. Acting as the α2M* cell surface signaling receptor, GRP78 regulates the Gs-mediated cAMP production and the pro-inflammatory COX-2-PGE-cAMP signaling cascade[100,101]. Regulatory T cells (Tregs) are a subpopulation of T cells that drive immune suppression. In some cancers, increased numbers of Tregs promote cancer progression by active suppression of the immune responses against the tumour. Cell surface GRP78 in T cells forms a complex with and confers stabilization to cell surface TGF-β which is an immune regulator and inducer of Tregs[102]. Some cancer cells secrete GRP78, which modulates human monocyte differentiation into mature dendritic cells and subsequent recruitment of T cells leads to generation of Tregs[103].

GRP94 has an important role in immunity by facilitating MHC class I molecule-mediated antigen presentation; by inducing the maturation and activation of various cells involved in innate and adaptive immune responses; and by secretion of proinflammatory cytokines[20]. GRP94 is the unique and obligatory chaperone of Toll-like receptors (TLRs), facilitating their maturation and translocation to the cell surface. Macrophage-specific knockout of *Grp94* resulted in lack of response to TLR ligands and loss of innate immune function[104]

(Supplemental Table 2), and exhibited reduced colitis and inflammation-associated colon tumorigenesis[54]. Thus, GRPs regulate inflammation and immunity in both tumour cells and through interactions with the tumour microenvironment (Figure 2).

Stem cell regulation

The notion that cancers are perpetuated by a small population of tumour initiating cells (TICs) that exhibit stem cell-like properties suggests a link between deregulated stem cell activation and cancer development. Initially identified for leukemia, TICs have also been implicated in solid tumours. Hematopoietic stem cells (HSCs) must maintain a balance between quiescence and activation to respond to demands for hematopoiesis yet sustaining long-term stem cell maintenance. Consistent with the pro-survival properties of GRP78, acute inducible ablation of GRP78 in the adult hematopoietic system resulted in intrinsic reduction of the HSC pool through increased apoptosis[34] (Supplemental Table 2). Inactivation of PTEN in bone marrow HSCs led to activation of the PI3K-AKT pathway, expansion of the HSC population, development of a myeloproliferative disorder and eventually leukemia[105]. Strikingly, heterozygous knockdown of *Grp78* in *Pten*-null mice was sufficient to inhibit PI3K-AKT activation, restore the HSC population to a normal level and suppress leukemic blast expansion[48] (Table 1). This effect is mediated at least in part by GRP78 at the cell surface, as treatment of the *Pten*-null mice with a GRP78 targeted antibody also suppressed AKT activation and leukemic blast formation[17]. Despite their similarity as ER chaperones, acute loss of GRP94 in the bone marrow led to AKT activation and expansion of HSCs, corresponding with loss of surface integrin β4 expression and HSC niche attachment[106,107] (Supplemental Table 2). These findings provide the first evidence that GRPs regulate HSC homeostasis through distinct pathways with different outcomes.

In head and neck TICs, expression of GRP78 at the cell surface is associated with self-renewal, suppression of differentiation and radioresistance[108,109]. RNAi-mediated silencing of GRP78 suppressed the growth of head and neck TICs in a mouse xenograft model, suggesting that cell surface GRP78 is a novel biomarker of TICs and a potential therapeutic target[108,109]. PTEN inactivation, which occurs in about half of all cases of human liver cancer, results in steatosis, liver injury and inflammation, which lead to liver progenitor cell (LPC) proliferation and the development of liver cancer[110]. Reduction of GRP78 to less than 25% by genetic knockout in the mouse liver resulted in steatosis, but did not trigger LPC activation or malignancies[111,112] (Supplemental Table 2). However, a similar reduction in GRP78 expression in the *Pten*-null liver model increased steatosis and liver injury and accelerated hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) formation[111] (Table 1). Strikingly, intense GRP78 re-expression was observed in the cancer lesions, and GRP78 expression in the surrounding liver tissue also reverted back to the wild-type level[111], suggesting repopulation of the liver by GRP78 positive, unrecombined cells conferring survival advantage, as reported for other tissues[35]. Knocking out *Grp94* in the liver caused only mild injury, but in the *Pten*-null mice loss of *Grp94* perturbed cell adhesion, stimulated LPC proliferation and accelerated HCC and CC progression[113] (Table 1). In human liver cancer, as well as in the PTEN-null mouse model, the levels of GRPs are upregulated and correlate with poor prognosis (Supplemental Table 1). So how can these observations be reconciled? One plausible explanation is that in organs where loss of the

GRPs leads to progenitor cell activation, when coupled with other carcinogenic events, tumorigenesis may be accelerated. However, there is generally a gain rather than a loss of GRP function in cancer due to stress-induced expression of GRPs. Under these conditions, the GRPs, with their pro-proliferation and anti-apoptotic functions, protect tumour cells from the host defense systems and promote tumour progression and resistance.

GRPs in therapeutic resistance

The expression of GRPs, in both tumour cells and the stromal cells, as an adaptive response to stress induced by cancer treatments, could represent a major obstacle to therapeutic efficacy[5,26,77,114]. GRP78 has been extensively documented to confer resistance against a wide range of therapies including chemotoxic drugs, anti-hormonal agents, DNA damaging agents, anti-angiogenesis drugs and chromatin-modifying drugs, as well as radiation therapy[20,21,115-117]. The effects are observed in proliferating and dormant cancer cells, TICs, as well as in tumour associated endothelial cells, and involve not only the ER form of GRP78, but also the stressed-induced cytosolic isoform[63], the secreted form[118] and the cell surface form of GRP78[108]. Although less well-studied, GRP94 and GRP170 have been linked with chemoresistance in various tumours[71,84], and GRP75 has been linked with resistance to cisplatin in ovarian cancer[119].

Targeting GRPs

As the GRPs are crucial factors in the multiple steps of tumorigenesis and often induced in tumours that have developed resistance against conventional therapy, they are attractive targets for drug and vaccine development to combat cancer progression and recurrence. Recently, GRP inhibitors acting at multiple levels have been identified (Figure 3). Importantly, cell surface expression of GRPs primarily in malignant but not normal cells *in vivo* offers the opportunity for cancer-specific therapy and drug delivery without harming the normal organs. Additionally, because GRPs are upregulated in the cancer microenvironment, their promoters could be of use in the development of gene therapies for the treatment of cancer.

Inhibitors of the GRPs

In principle, agents that inhibit the synthesis, stability or activity of the GRPs can simultaneously suppress their function at various cellular locations. The challenge is to minimize toxicity to normal organs. Various heterozygous knockout mouse models revealed that a 50% decrease in GRP78 expression has no effect on normal organs but significantly impedes tumour growth and angiogenesis[89] (Table 1). This implies that agents that selectively block the stress induction of GRP78 will affect tumours that require a high level of GRP78 and spare normal organs. Natural compounds with anti-cancer properties that suppress GRP78 induction have been reported, however, they exert pleiotrophic effects[5,120]. Specific cancers also express microRNAs that can act cooperatively to suppress *GRP78* translation (Figure 3) and reverse chemoresistance[121]. However, specific inhibitors of *GRP78* stress induction remain to be identified.

Alternatively, as the *GRP78* promoter is highly active in aggressive solid tumours, this offers the opportunity to use the *GRP78* promoter to direct expression of suicide genes, immunosuppressors and tumour suppressors in anti-cancer therapy. As proof-of-principle, the *Grp78* promoter driving the expression of the herpes simplex kinase suicide gene in a retroviral system results in eradication of sizable tumours[122,123]. Recently, systemic administration of a dual tumour-targeted phage containing the RGD tumour homing ligand and the *Grp78* promoter, showed persistent transgene expression *in vitro* and significant killing of therapy resistant tumours *in vivo*[124]. Likewise, cancer-inducible transgene expression can be directed by the *Grp94* promoter in tumours of various origins and cancer-associated macrophages[125].

Selective destruction of GRP78 at the protein level might be possible owing to the discovery of a bacterial toxin SubAB which cleaves GRP78 at a single site (L416-L417) in the hinge region connecting the ATPase and the substrate binding domain of the molecule, thereby inactivating it[126]. Systemic delivery of an engineered fusion protein combining epidermal growth factor (EGF) and SubAB was toxic to EGFR expressing cancer cells *in vitro* and caused a delay in the growth of human breast, prostate and glioblastoma xenografts in mice[127,128]. One of these studies[128] also showed that modest cleavage of GRP78 in normal mouse liver cells due to EGFR expression did not lead to weight loss, consistent with the findings in genetic models that normal organs including the liver can tolerate partial decrease in GRP78 levels[39].

Since the ATPase catalytic activity of GRP78 is necessary for its anti-apoptotic function[60], targeting its ATP binding domain can effectively inactivate GRP78 in cancer. Several plant compounds including (-)epigallocatechin gallate (EGCG), honokiol and aspirin (also known as salicylate) directly bind to this domain and inhibit the ATPase activity (Figure 3), and this is associated with the sensitization of cancer cells to chemotoxic agents[129-131]. Furthermore, an unconjugated peptide derived from the co-chaperone BAG1 binds to the substrate binding domain of GRP78 and inhibits its protein refolding activity, and prostate cancer cells stably expressing this peptide showed reduced growth and apoptosis in xenograft models in a manner dependent on binding to GRP78[132]. In glioblastoma cells, GRP78 can also be inactivated via acetylation by vorinostat, a deacetylase inhibitor with anti-tumour activity[133].

Recently, specific inhibitors against GRP94 function have been identified based on its unique secondary nucleotide binding pocket[72,134]. One of the compounds, PU-WS13, has been shown to reduce the viability of breast cancer cells expressing high level of cell surface ERBB2[72] and human multiple myeloma cells[53] *in vitro*. Interestingly, honokiol induces calpain-mediated cleavage GRP94 in human gastric cancer cells, associating with apoptosis and reduction in tumour growth[135]. GPM1, a chemical that can bind GRP94, suppresses its surface presentation through increased ER retention. This chemical was shown to compromise the immune functions of GRP94 *in vivo*; however, its efficacy in cancer is not known[136]. MKT-077, a cationic rhodacyanine dye, binds the nucleotide-binding domain of GRP75, abrogates its interaction with p53 and reactivates the transcriptional and pro-apoptotic activities of p53 in cancer cells, but not in normal cells *in vitro*[137-139].

Additionally, virtual screening of a drug database has revealed several small molecule inhibitors that are able to interrupt the p53-GRP75 complex[140].

Collectively, these proof-of-concept studies demonstrate that GRP inhibitors can selectively confer toxicity to cancer cells *in vitro* and *in vivo*, warranting further development and validation.

Cytotoxic agents targeting cell surface GRP

Preferential expression of GRP78 on the surface of tumour cells *in vivo* enables specific tumour targeting with minimal harmful effects on normal cells[11,13,141]. As cell surface GRP78 expression is further detected in some TICs and increased in metastatic and drug resistant tumours[6,16,117] and in hypoxic endothelial cells supporting tumour growth[68,78], cytotoxic agents against cell surface GRP78 have the potential to target these cells in addition to the primary tumour.

Several synthetic peptides composed of GRP78 binding motifs fused to cell-death inducing peptides or cytotoxic drugs are able to promote apoptosis in cancer cells *in vitro*, including human prostate, and breast cancer cells, human melanoma, chemotherapy-resistant B lineage acute lymphoblastic leukemia cells and multidrug resistant gastric cells[6,142-148] (Figure 3). Furthermore, xenograft and isogenic mouse models were used to validate the efficacy of the peptides in suppressing tumour growth of human prostate and breast cancer, melanoma, as well as bone metastasis with no apparent toxicity[6,142,145]. The GRP78 binding peptides have been conjugated to nanoparticles or liposomes for more efficient drug delivery[145,149], and such agents are able to home to endothelial cells in tumours, suppressing growth and prolonged survival of colon carcinoma bearing mice[82]. Furthermore, a reconstructed protein containing GRP78 binding peptide and mung bean trypsin inhibitor displays targeted anti-cancer effects both in vitro and in vivo in colorectal cancer[150].

Recently, MAb159, a high affinity GRP78 specific mouse monoclonal IgG antibody was identified that triggers endocytosis and degradation of cell surface GRP78, and activates both intrinsic and extrinsic apoptosis[17]. MAb159 causes cancer cell death and suppresses the growth of colon and lung xenografts, the metastatic growth of human breast and melanoma xenografts and the growth of prostate cancer and leukemia in genetically engineered mouse models, at least in part through inhibition of the PI3K signaling pathway[17]. MAb159 also synergizes with Irinotecan, a topoisomerase I inhibitor, in suppressing human colon cancer xenograft growth. A humanized MAb159 retains anti-tumour activity with no toxicity in mice and exhibits favorable pharmacokinetics[17]. In principle, this antibody can also be used as an *in vivo* imaging agent for selection of patients expressing cell surface GRP78 and to determine whether that predicts disease progression and response to therapy. Another screen yielded a mouse monoclonal IgG antibody targeting the carboxyl-terminal domain of GRP78, C107, which is capable of inducing apoptosis in melanoma cells *in vitro* and slowing their growth as xenografts in mice[151]. A human monoclonal IgM antibody (PAT-SM6) isolated from a patient with gastric cancer that can simultaneously bind low density lipoproteins and multiple GRP78 molecules on the surface of tumour cells, induces lipid accumulation and apoptosis in human multiple myeloma cells[152,153], and suppresses human melanoma growth both *in vitro* and in xenografts[154]. Based on favorable safety profiles in

phase I studies, the efficacy of PAT-SM6 is being tested in clinical trials[154]. It has also been reported that autoantibodies against GRP78 from ovarian cancer patients promote apoptosis and decrease the invasiveness of ovarian cancer cells[155]. In another study, autoantibodies against GRP78 from prostate cancer patients trigger ER Ca^{2+} release in human bladder carcinoma cells and increase tissue factor procoagulant activity, implying that blocking cell surface GRP78 signaling could potentially reduce the risk of cancer-related thrombotic events[156]. As proof-of-principle that cell surface GRP75 may also be amendable to therapy, intra-tumoural and intra-peritoneal injections of an anti-GRP75 antibody results in tumour growth suppression[22].

Vaccination strategies

Molecular chaperone preparations from tumours carrying tumour antigens offer a personalized, polyvalent vaccine therapy[2,23,157]. Although vaccination of lethally irradiated cancer cells expressing autologous secretory GRP94 fusion proteins protected mice from primary tumour growth and metastasis[158], vitespen, a GRP94-peptide complex that was purified *ex vivo* from individual patient's tumour cells showed variable immunogenicity and overall limited efficacy in clinical trials, with clinical responses only in certain patient subsets[159-161]. Recent studies showed that low dose of GRP94 immunization activated cytotoxic T lymphocytes with some tumour suppression in mice whereas high dose induced Treg proliferation and immune suppression, in a manner dependent on TLR-mediated NF-κB activation[162]. The use of GRP94 fusion proteins with tumour antigens, depletion of Treg cells, and pooled GRP94 vaccines have all been proposed to enhance the anti-tumour activity of GRP94 immunization[163], however challenges remain with these approaches.

GRP170 can form complexes with full-length protein antigens, such as gp100, and increase their presentation to immune cells, thereby augmenting multivalent T-cell-mediated anti-tumour immune responses[164]. Genetic modification of various poorly immunogenic melanomas to express a secretable form of GRP170 significantly suppressed tumour growth *in vivo*, and this was associated with increased tumour-infiltrating $CD8^+$ T cells and stimulation of dendritic cells[165]. GRP170-secreting tumour cells used as a cell-based vaccine is effective in treating established mouse prostate tumours[166]. Incorporation of a pathogen-associated molecule such as the NF-κB-activating domain of the bacterial flagellin into GRP170 maintains high efficient antigen-holding ability and through pathogen sensing TLR signaling, additionally activates dendritic cells, mounting a superior anti-tumour immune response against the primary tumour and distant metastasis[167]. The tumour derived secreted form of GRP78 is also able to elicit an anti-tumour immune response in mouse models as a result of activation of cytotoxic T cells[168].

Conclusions and perspectives

The GRPs possess functions that are distinct from the HSPs, impacting both the tumour cells and the tumour microenvironment. As the stress induction of GRPs could be a major contributor for tumorigenesis and therapeutic resistance, their specific inhibitors and targeting agents hold great therapeutic promise. Their clinical efficacies, as well as large GRPs as vaccines, warrant vigorous testing in the clinical setting. However, answers to key issues on basic mechanisms, such as how the stress-induced relocalization of the GRPs from

the ER to the cell surface and other organelles occurs, what their interactive partners are and the mechanisms of signaling, as well as the utility of GRPs as prognostic markers and companion imaging agents for precision cancer care will greatly advance the understanding of GRP biology and their applications in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1

Discovery of the GRPs

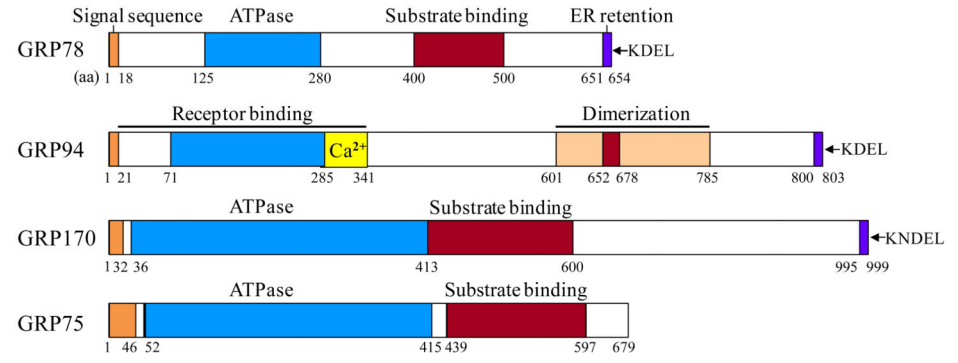
The GRPs were discovered in the mid-1970s as constitutively expressed cellular proteins induced by glucose starvation or a block in protein glycosylation, hence they were named glucose regulated proteins[169-171]. GRP78, encoded in humans by HSPA5, shares 60% amino acid homology with HSP70, including the ATP binding domain required for their ATPase catalytic activity (see figure) and is a HSP70 analogue in the ER. GRP78 is identical to BiP, originally discovered as an Ig heavy chain binding protein[172-174]. This led to the designation of GRP78 as an ER molecular chaperone, and it is now established as a ubiquitous protein essential for processing a wide repertoire of client proteins and maintaining the structural integrity of the ER[1,32,175].

Following the discovery of hamster GRP94 in 1984[176], GRP94 has been identified as endoplasmin (discovered as a Ca^{2+} binding protein)[177], ERp99 (discovered as a major ER glycoprotein)[178], and as the tumour rejection antigen gp96[179]. GRP94 encoded in humans by HSP90B1, shares 50% amino acid homology with HSP90 and is one of four HSP90 isoforms[180]. As well as being an ER chaperone, GRP94 is also a regulator of innate and adaptive immunity[1,4,181].

GRP75 encoded in humans by HSPA9 was first identified as a 66 kDa protein ($p66^{mot-1}$) linked to mortality with anti-proliferative properties[182]. cDNA cloning and a homology search revealed 80% homology to yeast mitochondrial HSP and 70% homology with mouse HSP70 (HSPA1A). Although GRP75 can localize to multiple subcellular sites, its primary location is in the mitochondria, as directed by its N-terminal leader sequence[3] (see figure).

Studying proteins induced by glucose starvation led to the discovery of a 150 kDa protein, GRP170[183]. GRP170, encoded in humans by HYOU1, is a large HSP70/HSP110-like protein in the ER[184] that is induced by hypoxia[185,186] (see figure).

Therefore, all of the GRPs can function as chaperones that can be induced during cellular stress.



Box 1 Figure Legend (Optional). Functional domains of the GRPs

The locations of the signal sequence targeting the proteins into the ER (GRP78, GRP94 and GRP170) or the mitochondria (GRP75) are shown. The ATPase and substrate

binding domains are indicated for all the GRPs. The location and the ER retention motifs for GRP78, GRP94 and GRP170 are shown. The Ca^{2+} binding, receptor binding and dimerization domains for GRP94 are denoted.

Box 2

Transcriptional activation of *GRP* promoters

The GRP78 promoter contains three ER stress response elements (ERSE) located upstream of the TATA element[187,188]. In non-stressed cells, NF-Y (also known as CBF), SP1 and histone deacetylase 1 (HDAC1) bind to the ERSEs and maintain GRP78 at a low basal transcriptional level[189]. Upon ER stress, ATF6 dissociates from GRP78 in the ER and translocates to the Golgi where it is cleaved to generate a form of ATF6 that can enter the nucleus [ATF6(N)] (see Figure 1). ATF6(N), which binds to the ERSE through binding to NF-Y, also associates with YY1 and increases the binding of YY1 to the GRP78 promoter[189]. YY1 associating protein arginine methyltransferase (PRMT) and the histone transacetylase p300 are also recruited to the GRP78 promoter, concurrent with histone 4 acetylation and arginine 3 methylation known to activate transcription[189]. Together, these transcription factors and chromatin modifiers form an ERSE binding complex, which can include the transcription factor TFII-I that functions as a scaffold protein[190]. While the IRE1/XBP-1 is also an important branch of ER stress, MEFs devoid of XBP-1 showed only modest effect on ER stress induction of GRP78 and no effect on GRP94[191]. Thus the XBP-1 may contribute to GRP transcription but is not obligatory. ER stress induction of GRP78 might also be partly attributed to ERSE-independent pathways, mediated by ATF4, a bZIP transcription factor, in complex with cAMP-responsive element-binding protein 1 (CREB1)[30]. Promoters of GRP94, GRP75 and GRP170 contain the ERSE consensus sequence and are similarly regulated[4,25,125,192].

Box 3

GRP interconnectivity in the ER and mitochondria

In addition to protein folding and secretion, the ER is central to Ca^{2+} homeostasis and the regulation of apoptosis. As low affinity, high capacity Ca^{2+} binding proteins, GRP78 and GRP94 help maintain ER Ca^{2+} balance[1]. Mitochondria are the site of oxidative phosphorylation-dependent ATP generation that is critical for maintaining energy homeostasis, and they also integrate and transduce apoptotic signals, and participate in the regulation of intracellular Ca^{2+}. Structural and functional analyses reveal zones of close contact between the ER and mitochondria, referred to as mitochondria associated membranes (MAMs)[193]. MAMs enable the efficient transmission of Ca^{2+} from the ER to mitochondria and molecular chaperones such as GRP75, calnexin, calreticulin, ERp44, ERp57 and the sigma-1 receptor coexist at MAMs[194]. Signaling from the ER to mitochondria can be critical in the induction of mitochondrial dependent cell death pathways[61,195,196]. The UPR promotes GRP78 localization to the mitochondria[64,197] and slows the increase of Ca^{2+} in mitochondria after stress and reduces free radical generation, associating with protection against ischemic injury[196]. For cancer cells, over-expression of GRP78 might protect them against damage resulting from potentially carcinogenic free radicals generated from endogenous or exogenous source. GRP78 might also regulate mitochondria energy balance through modulation of GRP75 and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) expression[198]. In specific tissues, GRP78 haploinsufficiency leads to compensatory upregulation of GRP75, GRP94 and other ER chaperones[33,198]; conversely, GRP94 deficiency triggers GRP78 upregulation[199].

Collectively, these findings demonstrate the interconnectivity of the GRPs and adaptive responses in cells to maintain functional Ca^{2+} and ATP homeostasis in the ER and mitochondria. These mechanisms might result in a survival advantage to cancer cells in the face of the variety of metabolic and environmental perturbations that exist in growing tumours.

Key points NRC-12-276V4

1. The glucose regulated proteins (GRP78, GRP94, GRP170 and GRP75) are members of the heat shock protein family primarily residing in the endoplasmic reticulum (ER) or the mitochondria and they are induced at the transcriptional level upon ER stress.
2. As molecular chaperones, the GRPs regulate protein quality control and degradation, with GRP78 serving additionally as a pivotal regulator of the unfolded protein response and the apoptotic machinery associated with the ER.
3. The GRPs can be actively translocated to other cellular locations and secreted, and assume additional functions that control cellular signaling, proliferation, invasion, apoptosis, inflammation and immunity, which have major implications in cancer progression and therapeutic resistance.
4. Specific roles of GRPs in development, tumorigenesis, metastasis and angiogenesis have been identified in vitro and validated in genetically engineered mouse models.
5. GRP overexpression is widely reported in many human cancers and associated with aggressive properties, suggesting potential prognostic value and that interfering with their production or activities in those tumors might provide new approaches for anti-cancer treatment.
6. The discovery that cell surface GRP78 is preferably expressed in cancer and stressed endothelial cells leads to the development of therapeutic agents specifically targeting cell surface GRP78 capable of inducing cancer cell apoptosis and suppressing tumorigenesis with minimal toxicity.
7. While the GRPs are attractive targets for drug development, they can also serve as mediators for cancer specific drug delivery, transcriptional targeting of cancer and vaccine development.
8. The large chaperone GRP170 with superior property in presentation of full-length protein antigens opens up a new vaccine platform to augment anti-tumor immune responses.

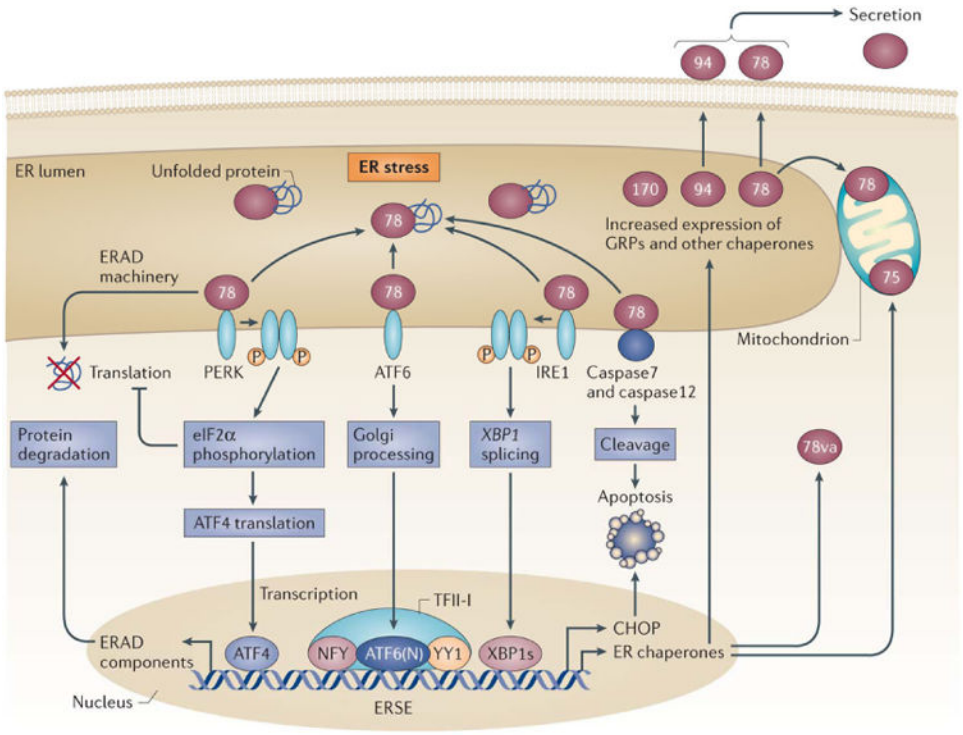


Figure 1. GRPs in UPR and the stress response

ER luminal GRP78 acts as a UPR signaling regulator by binding to and maintaining the ER stress sensors (PERK, ATF6 and IRE1) in inactive forms. It also binds to and suppresses the activation of ER associated caspase-7 and -12 (C7/12). Upon ER stress, GRP78 is titrated away through binding to mal-folded proteins. This triggers the UPR, as exemplified by dimerization of PERK and IRE1, and activation of their downstream signaling pathways, leading to arrest of translation and ER associated protein degradation (ERAD). The UPR also generates the active nuclear form of ATF6, as well as ATF4, and the spliced form of XBP (XBP-1s), which act in concert with other transcriptional factors including YY1, NF-Y and TFII-I and chromatin modifiers, to activate the ER stress response element (ERSE) present on the promoters of ER stress responsive genes. A major UPR response is to induce the transcription of ER folding proteins such as the GRPs to booster the ER protein folding capacity, as well as the mitochondrial (Mito) chaperone GRP75. Stressed cells actively promote re-localization of GRP78 and GRP94 to the plasma membrane (PM), and in some instances, their secretion, and generate a cytosolic isoform of GRP78 (78va) through alternative splicing. Nonetheless, UPR can also induce transcription of the pro-apoptotic transcription factor *CHOP*, and following release from GRP78, caspase-7 and caspase-12 are activated, triggering apoptosis. Thus, the UPR regulates the balance between survival and cell death in stressed cells, and the up-regulation of the GRPs represents a major adaptive, protective measure through maintenance of cellular homeostasis.

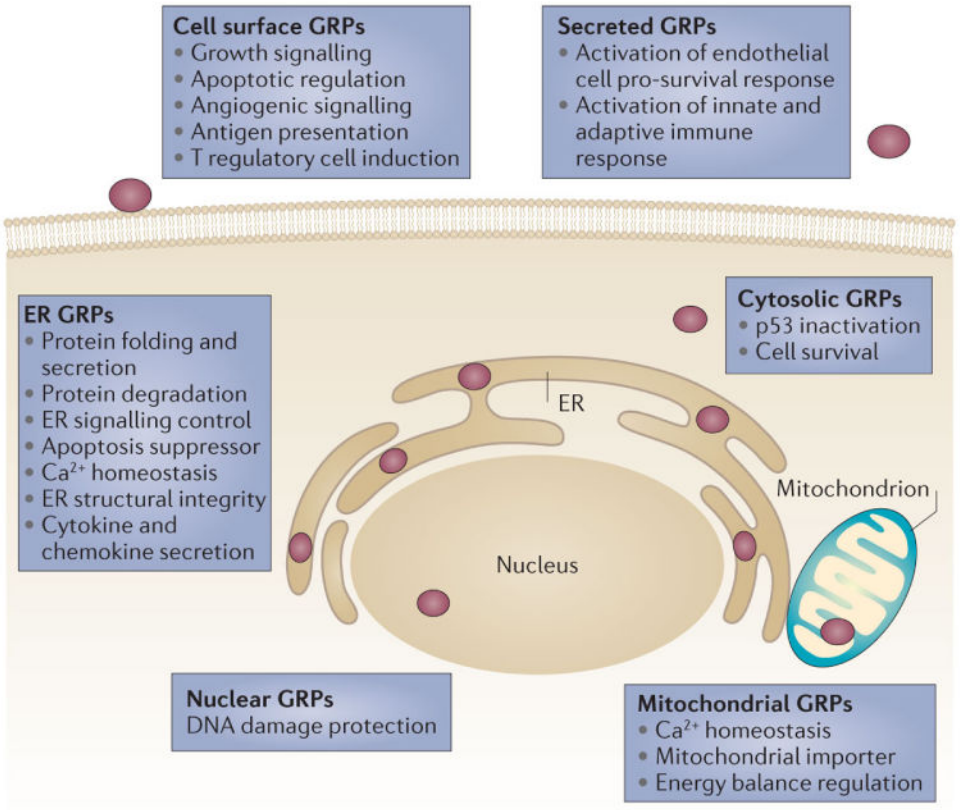


Figure 2. GRPs in survival and immunity

The majority of GRP78, GRP94 and GRP170 are located in the ER lumen serving as ER folding proteins, and GRP75 is primarily a mitochondrial chaperone. Under ER stress or pathological stress conditions, a subfraction of GRP78 and GRP94 translocate to the cell surface and their secreted forms can be detected. Cell surface GRPs control critical growth and apoptotic signaling functions, as well as immune functions notably antigen presentation. ER stress also induces GRP78 translocation into the nucleus and mitochondria, and alternative splicing of *GRP78* mRNA leading to the generation of a cytosolic isoform. The pro-survival functions of the GRPs in the various subcellular locations are indicated in blue, and the immune functions in red.

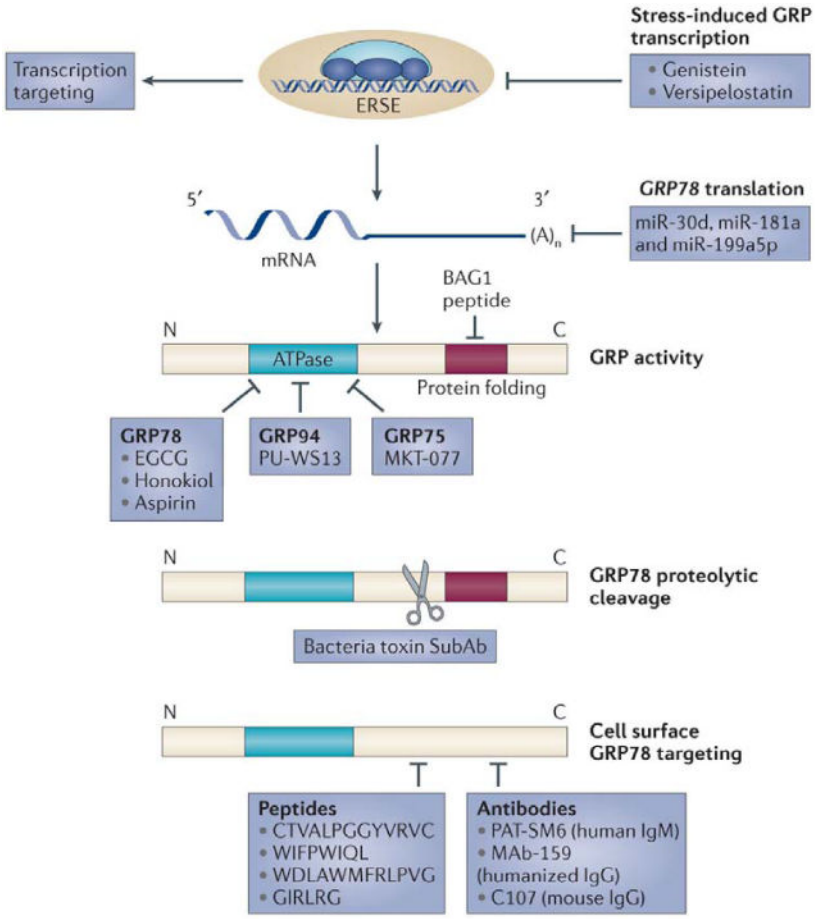


Figure 3. Summary of agents targeting the GRPs

Stress-induced GRP expression can be suppressed at the transcriptional level by inhibiting transcription factors required for the stress induction of the *GRP* promoter. Several microRNAs have been identified that suppresses the translation of *GRP78* mRNA in cancer cells. Since chaperone function of the GRPs depends on the ATPase catalytic activity, compounds or peptides that bind to their ATP binding domains or alter their ATPase activity are effective in suppressing GRP function. A BAG1 peptide binds to the GRP78 substrate binding domain and inhibits its protein refolding activity. GRP78 can be cleaved specifically by the bacterial toxin SubAB, which renders it non-functional. Cell surface GRP78 can be effectively targeted by specific peptides in conjugated or non-conjugated forms and the human plasminogen factor Kringle 5 (K5). Antibodies against cell surface GRP78 are able to suppress GRP78-mediated oncogenic signaling and induce cancer cell death by multiple mechanisms. On the other hand, the inducible *GRP* promoter containing the ER stress responsive elements (ERSEs) can be used to direct cytotoxic gene expression in cancer cells. The GRP inhibitors listed have shown a wide range of anti-cancer effects *in vitro* and *in vivo*.

Table 1
Role of GRP78 and GRP94 in mouse models of cancer

Cancer Type	Mouse Genotype	KO	Key Phenotypes	Refs.
Breast carcinoma	*Grp78*$^{+/-}$; MMTV PyVT	+/-	Prolongs latency period, impedes tumour growth Tumour cell proliferation↓, angiogenesis↓, apoptosis↑	39
Prostate adenocarcinoma	*Grp78*$^{f/+}$ or *Grp78*$^{f/f}$; *Pten*$^{f/f}$; *probasin Cre*	+/- or -/-	Suppresses tumorigenesis AKT activation↓	47
Leukemia	*Grp78*$^{f/+}$; *Pten*$^{f/f}$; *Mx1-Cre*	+/-	Suppresses leukemic blast cell expansion PI3K/AKT signaling↓ Normal hematopoietic phenotype	48
Liver cancer	*Grp78*$^{f/f}$; *Pten*$^{f/f}$; *Alb-Cre*	-/-	Exacerbates steatosis and liver injury Liver progenitor cell activation, accelerates tumorigenesis Strong GRP78 re-expression in cancer lesions	111
	Grp94$^{f/f}$; *Pten*$^{f/f}$; *Alb-Cre*	-/-	Minor liver injury, disrupts cell adhesion protein organization Liver progenitor cell proliferation↑ ERK activation, accelerates tumorigenesis	113
Multiple myeloma	*Hsp90b1*$^{f/f}$*; CD19-Cre; XBP1-sTg*	-/-	Suppresses tumour growth Inhibits Wnt/β-catenin signaling	53
Colorectal cancer	*Hsp90b1*$^{f/f}$*; LysM-Cre*	-/-	Reduction in number and size of colitis-associated colon cancer Colonic epithelial β-catenin mutation↓,Wnt signaling↓	54