

## REVIEW

# The critical roles of endoplasmic reticulum chaperones and unfolded protein response in tumorigenesis and anticancer therapies

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Cancer progression is characterized by rapidly proliferating cancer cells that are in need of increased protein synthesis. Therefore, enhanced endoplasmic reticulum (ER) activity is required to facilitate the folding, assembly and transportation of membrane and secretory proteins. These functions are carried out by ER chaperones. It is now becoming clear that the ER chaperones have critical functions outside of simply facilitating protein folding. For example, cancer progression requires glucose regulated protein (GRP) 78 for cancer cell survival and proliferation, as well as angiogenesis in the microenvironment. GRP78 can translocate to the cell surface acting as a receptor regulating oncogenic signaling and cell viability. Calreticulin, another ER chaperone, can translocate to the cell surface of apoptotic cancer cells and induce immunogenic cancer cell death and antitumor responses *in vivo*. Tumor-secreted GRP94 has been shown to elicit antitumor immune responses when used as antitumor vaccines. Protein disulfide isomerase is another ER chaperone that demonstrates pro-oncogenic and pro-survival functions. Because of intrinsic alterations of cellular metabolism and extrinsic factors in the tumor microenvironment, cancer cells are under ER stress, and they respond to this stress by activating the unfolded protein response (UPR). Depending on the severity and duration of ER stress, the signaling branches of the UPR can activate adaptive and pro-survival signals, or induce apoptotic cell death. The protein kinase RNA-like ER kinase signaling branch of the UPR has a dual role in cancer proliferation and survival, and is also required for ER stress-induced autophagy. The activation of the inositol-requiring kinase 1 $\alpha$  branch promotes tumorigenesis, cancer cell survival and regulates tumor invasion. In summary, perturbation of ER homeostasis has critical roles in tumorigenesis, and therapeutic modulation of ER chaperones and/or UPR components presents potential antitumor treatments.

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## INTRODUCTION

The endoplasmic reticulum (ER) is a perinuclear, cytosolic compartment essential for the synthesis, folding and modification of secretory and membrane proteins. It is also the site for lipid synthesis and a major intracellular site for Ca<sup>2+</sup> storage. Physiological and pathological conditions that perturb the ER, such as nutrient deprivation, hypoxia, ER Ca<sup>2+</sup> depletion, impaired glycosylation or disulfide bond formation, oxidative stress and viral or bacterial infection, may lead to ER stress. ER stress occurs when the protein load exceeds the ER capacity to fold or degrade them, and is manifested by the accumulation of misfolded proteins in the ER. ER stress triggers an evolutionarily conserved quality control mechanism, the unfolded protein response (UPR), which aims at restoring ER homeostasis by activating a cascade of signaling molecules to transiently arrest protein translation, to induce ER molecular chaperones and enzymes that enhance the protein folding capacity, and to initiate a process to export and degrade the misfolded ER proteins.<sup>1,2</sup> The most abundant and well-characterized ER chaperone proteins include glucose regulated protein (GRP) 78,

GRP94, calreticulin (CRT) and protein disulfide isomerase (PDI). Because of increased protein synthesis in proliferating cancer cells, tumor cells require increased ER capacity and function. ER chaperones serve a host of important roles in maintaining ER homeostasis contributing to cancer cell survival and progression. For example, GRP78 is critical for tumorigenesis and therapeutic resistance. Recently, much progress has been made in identifying diverse roles for ER chaperones in cancer. Because of the interconnectivity of the ER with other cellular compartments, it is now becoming clear that chaperones traditionally thought to remain in the ER, can function beyond this compartment and are involved in processes beyond protein folding and posttranslational modification. In particular, the cell surface form of GRP78, GRP94, CRT and PDI assume novel signaling functions that regulate proliferation, apoptosis and immunity. In view of the many excellent general reviews on the UPR, this review focuses on the diverse functions of major ER chaperones inside and outside of the ER and updates the contribution of the UPR signaling pathways within the context of cancer progression and anticancer therapy.

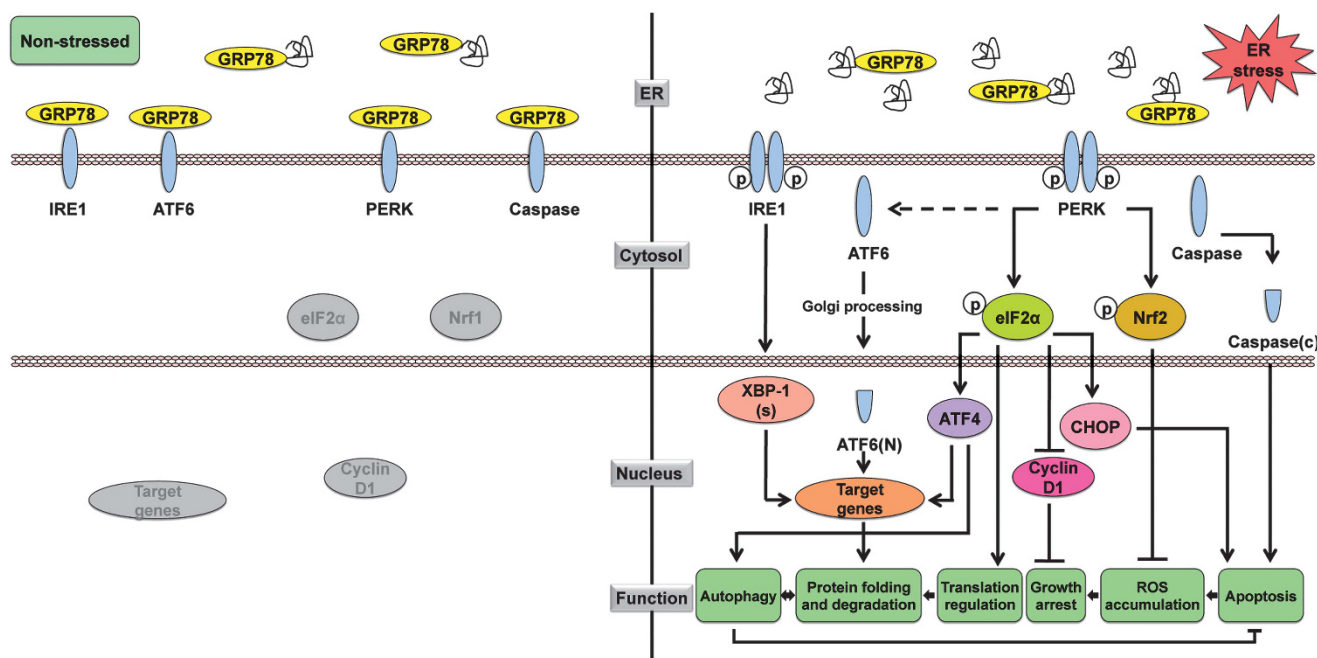
## ER HOMEOSTASIS AND THE UPR

There are three canonical branches in the UPR signaling pathway, which are mediated by three ER stress sensors: protein kinase RNA-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1 $\alpha$ ), and the activating transcription factor 6 (ATF6). A major ER chaperone, the 78 kDa GRP78, also referred to as BiP/HSPA5, acts as a master regulator of the UPR through direct interaction with all three sensors and maintains them in an inactive form in non-stressed situations (Figure 1).<sup>3–5</sup> Upon ER stress, GRP78 is titrated away by the accumulated misfolded proteins, releasing the UPR sensors, which allows the activation and transduction of UPR signals across the ER membrane to the cytosol and the nucleus. PERK is an ER transmembrane protein with an ER luminal stress-sensing domain and a cytosolic kinase domain. An important function of PERK in the UPR is to facilitate the attenuation of global protein synthesis via the phosphorylation of eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which suppresses 80S ribosome assembly. This results in the inhibition of cyclin D1 translation and cell-cycle arrest.<sup>6</sup> Although global translation is suppressed under conditions of eIF2 $\alpha$  phosphorylation, select mRNAs containing regulatory sequences in the open reading frame in 5'-untranslated regions require the phosphorylation of eIF2 $\alpha$  for translation. The transcription factor ATF4 is one example and the translational upregulation of ATF4 can induce the expression of UPR target genes that promote ER folding capacity and adaptation to stress.

IRE1 $\alpha$  is a transmembrane Ser/Thr protein kinase that also has site-specific endoribonuclease (RNase) activity. Upon ER stress, IRE1 $\alpha$  dimerizes and autophosphorylates, and thereby activates its RNase activity to cleave a 26 base intron from the mRNA encoding

X-box-binding protein 1 (XBP1), resulting in a translational frame-shift and a translation of a spliced form of XBP1 (XBP1-s), which is a more stable and potent transcription factor of target genes including *DnaJ*, *p58*, *ERdj4*, *EDEM* and *PDI*, all involved in protein folding and ER-associated degradation.<sup>7,8</sup> ATF6, a basic leucine zipper transcription factor, when released from GRP78 upon ER stress, translocates from the ER to the Golgi apparatus, where it is cleaved by S1P and S2P proteases to generate the active nuclear form of ATF6 (p50). Cleaved ATF6 and spliced XBP1 act in parallel to mainly induce the transcription of genes encoding ER chaperones and enzymes that facilitate protein folding and maturation. Interestingly, although the PERK/p-eIF2 $\alpha$ /ATF4 pathway is canonically regarded as the translational controlling arm of the UPR, a recent report suggests that it is also required for the activation of ATF6 and its target genes, thereby fully integrating the regulatory networks of the UPR.<sup>9</sup>

In addition to these ER stress pathways, autophagy is activated upon ER stress and has been implicated as a defensive mechanism for maintaining ER homeostasis that promotes survival.<sup>10,11</sup> Autophagy is an intracellular protein degradation system required for the normal turnover of cellular components and for the starvation response. When autophagy is induced, a double-membrane structure called autophagosome is formed *de novo* or from existing membranes, possibly from the ER, to enclose the subcellular components. Loss of the integrity of the ER, for example, through depletion of GRP78, suppresses autophagosome formation induced by ER stress as well as nutrient starvation.<sup>12</sup> How might autophagy relieve ER stress? As shown in the yeast system, upon ER stress when the ER-associated degradation system is saturated, autophagy removes both



**Figure 1.** Unfolded protein response and its regulation on cell activities. Left panel: under non-stress condition, ER luminal GRP78, in addition to folding proteins, binds to IRE1 $\alpha$ , ATF6, PERK and caspase-12 and -7, quenching their activation. Right panel: when cells are under ER stress, GRP78 is titrated away through binding to the misfolded proteins, resulting in activation of the IRE1 $\alpha$ , ATF6 and PERK signaling pathway. IRE1 $\alpha$  activates its RNase activity to cleave the mRNA of XBP1, resulting in a spliced form of XBP1 (XBP1-s). ATF6 translocates from the ER to the Golgi apparatus, where it is cleaved into the active nuclear form ATF6(N). PERK dimerizes and autophosphorylates, and thereby phosphorylates its two major substrates eIF2 $\alpha$  and Nrf2. eIF2 $\alpha$  phosphorylation attenuates global protein synthesis, and inhibits cyclin D1 translation through which contributes to cell-cycle arrest. The phosphorylation of eIF2 $\alpha$  also activates the transcription of ATF4. XBP1(s), ATF6(N) and ATF4 act in concert to induce transcription of target genes mediating protein folding and degradation. Autophagy is also triggered. Another function of phosphorylated eIF2 $\alpha$  is to activate CHOP, which promotes apoptosis. The phosphorylation of Nrf2 activates the expression of enzymes required for ROS quenching, and thereby inhibits ROS accumulation. Procaspase-12 and -7, upon released from GRP78, are cleaved into their activated forms triggering apoptosis.

soluble and aggregated forms of unfolded proteins.<sup>13</sup> It has also been suggested that autophagy counterbalances ER expansion in the face of continuously accumulating unfolded proteins.<sup>14</sup> On the other hand, the onset of autophagy upon ER stress requires the UPR. Studies using mouse embryonic fibroblasts with deficiency in various UPR stress sensors showed that ER stress-induced autophagy was inhibited.<sup>15–17</sup> Although the precise requirement of specific UPR pathways to induce autophagy under different stress conditions awaits further resolution, the UPR and autophagy are integrated processes having important roles in restoring homeostasis upon stress, counteracting apoptotic mechanisms (Figure 1).

Although UPR activation leads to adaptations that may sustain cell survival, under severe and prolonged ER stress conditions where the cells fail to restore ER homeostasis, the UPR activates pathways that lead to apoptotic cell death.<sup>2,3</sup> Such measures protect the organism by eliminating damaged cells beyond repair. ER stress-induced apoptosis can be carried out in a number of ways. Examples include the translocation of the death effectors BAX and BAK from the ER to the mitochondria triggered by ER  $\text{Ca}^{2+}$  flux, caspase activation (C-12 and C-4 in mice and human, respectively) by tumor necrosis factor receptor-associated factor-2 and the activation of c-Jun-N-terminal kinase by IRE1 via phosphorylation and the inactivation of the anti-apoptotic protein BCL-2. Additionally, the synthesis of BCL-2 can be suppressed by CHOP, downstream of the PERK-eIF2 $\alpha$ -ATF4 pathway. However, it has been proposed that in stressed cells the pro-apoptotic effect of CHOP may be attributed more to its activation of GADD34 that promotes eIF2 $\alpha$  dephosphorylation, thereby acting in a negative feedback loop that leads to excessive recovery of protein synthesis that exceeds the ER folding capacity.<sup>2</sup>

## ER STRESS IN TUMORIGENESIS

Neoplastic progression is a multistep process resulting from genetic alterations that drive the progressive transformation of normal cells into malignant states by overriding growth arrest or senescence controls, and at the same time, suppressing the pro-apoptotic signals. Rapidly proliferating cancer cells require increased ER activity to facilitate the folding, assembly and transport of membrane and secretory proteins, and are thereby subjected to ER stress. Because of inadequate vascularization and rapid growth, tumor cells encounter growth-limiting conditions such as hypoxia and nutrient deprivation. An inadequate supply of glucose affects protein glycosylation and the production of ATP, both of which could lead to the accumulation of unfolded proteins in the ER, resulting in ER stress. Thus, in response to ER stress, activation of the UPR has been observed in various tumors.<sup>18</sup> In the case of leukemic cells, retroviral infection, the pathological expression of fusion protein PML-RAR $\alpha$  and the accumulation of reactive oxygen species (ROS) can all induce ER stress.<sup>19,20</sup> The hypoxic bone marrow (BM) environment and the high glucose demands resulting from rapid proliferation also contribute to ER stress in leukemia cells. Indeed, ER stress signaling has been implicated in the spontaneous apoptosis of leukemia cells<sup>21,22</sup> and inducible heterozygous knockout of *Grp78* in the BM suppresses *Pten*-null-mediated leukemogenesis.<sup>23</sup>

During the early stages of tumor development, ER stress and the activation of the UPR could affect tumorigenesis in multiple ways (Figure 2). For example, ER stress promotes angiogenesis through stimulating vascular endothelial growth factor (VEGF) expression and secretion.<sup>24,25</sup> It also induces cancer cell dormancy through G1 arrest in response to decreased cyclin D1 downstream of PERK activation.<sup>26,27</sup> During tumorigenesis, the robust upregulation of GRP78 and other ER chaperones by the UPR can enhance the ER protein folding capacity and maintain ER homeostasis. Additionally, the anti-apoptotic property of GRP78

can also counterbalance the cell death pathways that are still functional in the cancer cells. This, coupled with the induced dormancy, dually protects the cancer cells from apoptosis and allows for recurrence once favorable growth conditions return. A recent report further suggests a novel role of the tumor ER stress response in promoting macrophage activation and inflammation in the tumor microenvironment.<sup>28</sup> Macrophages cultured in conditioned medium from ER stressed tumor cells became activated, and they undergo ER stress with upregulation of GRP78, GADD34, CHOP and XBP1 splicing, and were able to recapitulate, amplify and expand the proinflammatory response of tumor cells, favoring tumor progression (Figure 2).

Despite the protective effects of the UPR, severe, persistent ER stress leads to cell death. Thus, overactivation of the ER stress pathways in hypoxic tumor cells has been shown to render them more sensitive to proteasome inhibitors such as bortezomib, resulting in increased cytotoxicity.<sup>29</sup> Interestingly, HSP90-inhibitor IPI504, another agent known to enhance proteotoxic stress, induces tumor regression only when combined with rapamycin, by promoting irresolvable ER and oxidative stress that result in catastrophic ER and mitochondria damage.<sup>30</sup> Nonetheless, in cancer cells where mutations often inactivate their apoptotic potential, mild, chronic ER stress could be beneficial for cancer cell survival. In a reconstituted cell culture system, it was demonstrated that chronic ER stress can be a predominantly adaptive pathway, primarily by maintenance of expression of UPR targets that facilitate survival, in particular ER chaperones such as GRP78 by ATF6 activation.<sup>31</sup> At the same time, mild, chronic ER stress leads to intrinsic instabilities of mRNAs and proteins that promote apoptosis such as CHOP and GADD34 (Figure 2). Thus, the UPR can be structured to allow cells to avert death as they adapt and both posttranscriptional and posttranslational mechanisms influence this outcome. Nonetheless, considering the complexity and heterogeneity within the tumor as well as the microenvironment around the tumor, the interplay between UPR and the various signaling pathways will likely corporate to dictate whether apoptosis, growth arrest or proliferation will occur.

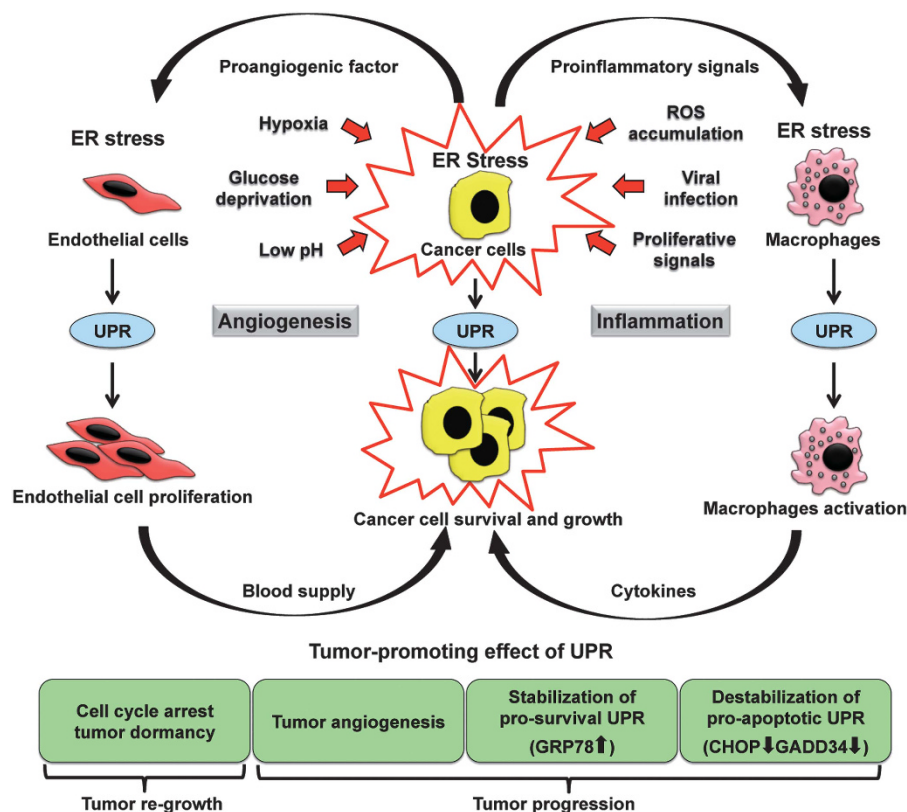
## THE PLEIOTROPIC ROLE OF THE ER MASTER CHAPERONE GRP78 IN CANCER

GRP78 is a multifunctional protein that can impact a wide range of human diseases via diverse mechanisms.<sup>4,5,32–34</sup> Although traditionally GRP78 is regarded as a lumen ER chaperone whose major function is to fold and process ER proteins, bind ER  $\text{Ca}^{2+}$  and maintain ER homeostasis, recent studies have established 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, where it binds interacting partners and exerts new effects on cell growth and signaling.<sup>35–37</sup> These discoveries change the paradigm on GRP78 function and offers novel therapeutic approaches to target GRP78 (Figure 3).

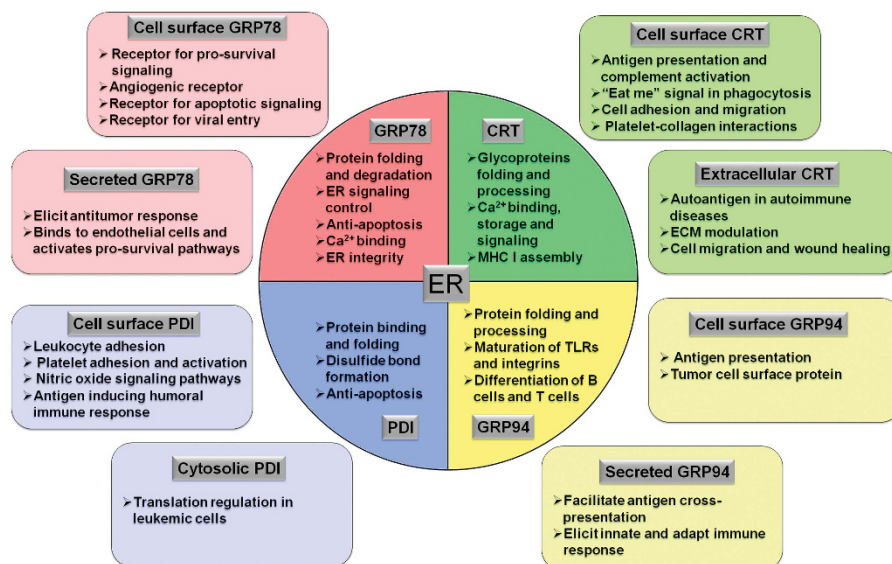
## GRP78 REGULATES CELL SURVIVAL AND PROLIFERATION

GRP78 is widely observed to be upregulated in cancer cells.<sup>4,5,33,38</sup> One major function of GRP78 is protection against stress-induced apoptosis. The ability of GRP78 to block apoptosis includes binding and inactivating pro-apoptotic components such as BIK and caspase-7 that localize to the ER, as well as suppressing the induction of CHOP, which mediates the apoptotic arm of the UPR.<sup>39,40</sup> Recently, a novel mechanism for the anti-apoptotic function of GRP78 has been uncovered involving functional interactions between GRP78, BIK, NOXA and BCL-2.<sup>41</sup> This new study showed that GRP78 is able to negate apoptosis resulting from BIK induction, even if it is assisted by another BH-3 protein such as NOXA. GRP78 binds to BIK in a BH-3 domain independent





**Figure 2.** The vicious circle of ER stress and unfolded protein response in promoting tumor progression. Cancer cells are under ER stress because of the growth signaling and factors from the microenvironments. Adaptive UPR is activated to support tumor cell survival and growth. Cancer cells under ER stress secrete pro-angiogenic factors to stimulate the proliferation of endothelial cells, which in return promotes cancer cell survival and tumor growth. Cancer cells under ER stress also secrete proinflammatory signals to the stromal cells in the vicinity, mostly tumor-associated macrophages, which in turn are activated and secrete inflammatory cytokines that promote tumor growth, angiogenesis, invasion and metastasis.



**Figure 3.** The different localizations and functions of major ER chaperones. The circle in the center indicates ER, where the majority of ER chaperones are located. The squares outside of ER indicate non-traditional localization of these chaperones and their functions.

manner through its amino portion, consistent with previous findings that a subfraction of GRP78 may exist in a transmembrane configuration with the amino portion exposed

to the cytosol.<sup>39,42</sup> Interestingly, GRP78 and BCL-2 form separate complexes with different domains of BIK, and increasing amounts of GRP78 expressed in cells leads to a reduction of BCL-2 binding

to BIK and vice versa.<sup>41</sup> These results suggest that when the expression of BIK is elevated under circumstances such as estrogen starvation or anti-estrogen treatment of breast cancer cells, it promotes the formation of BIK/BCL-2 heterodimers at the outer surface of the ER. The high ratio of BIK:BCL-2 changes the set of proteins BCL-2 interacts with the ER, leading to  $\text{Ca}^{2+}$  release and initiation of the apoptosis including the translocation of BAX to the mitochondria and the release of cytochrome c to the cytosol. However, when GRP78 is expressed at high levels in cancer cells because of ER/metabolic stress associated with long-term estrogen deprivation, GRP78 binds to and sequesters BIK through complex formation. With reduced binding to BIK, BCL-2 is able to suppress ER  $\text{Ca}^{2+}$  release, thereby suppressing apoptosis. This mechanistic explanation is consistent with another report implicating BCL-2 in the survival pathway activated by GRP78 overexpression.<sup>43</sup> Considering that the apoptotic function of BIK has also been implicated in response to chemotherapy and proteasome inhibitors,<sup>44,45</sup> this mechanism may also apply in these systems. In addition to being a regulator of apoptosis, GRP78 is also identified as a regulator of autophagy because of its role in maintaining ER integrity and homeostasis.<sup>12</sup>

Evidence is accumulating that GRP78 is critical for cell proliferation. Genetic knockout of *Grp78* leads to a much reduced embryonic cell proliferation as well as massive apoptotic death of the inner cell mass, leading to embryonic lethality at day E3.5.<sup>46</sup> This provides the first hint that GRP78 may be important for pluripotent cell homeostasis and survival. In adult cells, GRP78 is expressed at low levels in the normal adult brain but is highly elevated in malignant glioma specimens and human malignant cell lines, correlating with their rate of proliferation, and knockdown of GRP78 by small interfering RNA leads to a slowdown in glioma cell growth.<sup>38</sup> This is further supported by the observation that in an endogenous breast cancer model, GRP78 haploinsufficiency inhibits tumor cell proliferation.<sup>47</sup> How might GRP78 facilitate cell proliferation? This could be because of the ER chaperone function of GRP78 in growth factor secretion and/or the maturation of growth factor receptors. Another major discovery is that ER stress actively promotes the localization of GRP78 to the cell surface,<sup>48</sup> and selected cell types, notably cancer cells, express cell surface GRP78 that acts as a multifunctional receptor.<sup>36,49</sup> One function of cell surface GRP78 is to enhance PI3K/AKT signaling, leading to both increased proliferation and survival. It is recently reported that ligation of prostate cancer cell surface GRP78 by activated  $\alpha 2$ -macroglobulin ( $\alpha 2$ -M) upregulates the prostate-specific antigen, which is then secreted into the medium and binds  $\alpha 2$ -M. The resultant prostate-specific antigen/ $\alpha 2$ -M complex then binds to cell surface GRP78, causing activation of multiple kinase signaling pathways, coupling with an increase in proliferation and protein synthesis.<sup>50</sup> Additionally, GRP78 and Cripto can form a complex at the cell surface and collaborate to inhibit transforming growth factor  $\beta$ -signaling and enhance cell growth.<sup>51</sup> Cell surface GRP78 is also reported to complex with Cripto in adult hematopoietic stem cells (HSCs) and maintains them in the hypoxic endosteal niche in the BM.<sup>52</sup>

### GRP78 IS REQUIRED FOR SOLID TUMOR PROGRESSION

Fast proliferating solid tumors are characterized with increasing hypoxia, nutrient starvation and acidosis, factors that activate the UPR. Correspondingly, GRP78 has been reported to be upregulated in solid tumors in various organs including the breast, liver, gastric, esophagus, brain, prostate, head and neck and melanoma, correlating with aggressive tumor behavior and recurrence.<sup>5,18,33,38,53–57</sup> Although xenograft studies with knockdown of GRP78 supported its requirement for tumor formation and progression,<sup>58</sup> the pathophysiological role of GRP78 in tumorigenesis has been directly validated using the heterozygous *Grp78* mice and mouse models of conditional

knockout of the *Grp78* in specific tissues. These studies revealed that without affecting mouse growth rate, organ development and antibody production, GRP78 haploinsufficiency prolongs the latency period and retards the progression of the oncogene-induced mammary tumors in the MMTV-PyVT breast adenocarcinoma mouse model.<sup>47</sup> The underlying mechanisms for this suppression include lowered tumor proliferation, increased apoptosis and dramatically reduced tumor angiogenesis. Likewise, heterozygous or homozygous deletion of *Grp78* specifically in the mouse prostate epithelium suppresses prostate tumorigenesis mediated by a loss of the tumor suppressor gene *Pten* without affecting postnatal prostate development and growth.<sup>59</sup> Strikingly, AKT activation in the PTEN-null prostate epithelium was potentially suppressed by the loss of GRP78, and a similar suppression of stress-mediated AKT activation was observed in human prostate cancer cells where GRP78 was knocked down by siRNA. As *Pten* mutation and AKT activation are key drivers of human cancer, inactivation of GRP78 may represent a novel approach to block tumorigenesis resulting from loss of PTEN tumor suppression or activation of oncogenic AKT, or both.

Cancer initiating cells (CICs) with self-renewal capacity have recently been implicated as the root cause of cancer metastasis, therapeutic resistance and recurrence. GRP78 has been reported to be highly elevated in breast disseminated tumor cells, which shared similar biological properties of CICs.<sup>60</sup> In agreement, differential systemic analysis revealed elevated GRP78 expression in head and neck CICs.<sup>56</sup> This same study also reported that such cells with cell surface expression of GRP78 contain cancer stemness properties of self-renewal, differentiation and radio-resistance; whereas knockdown of GRP78 by shRNA promoted head and neck CIC differentiation and apoptosis and impaired their tumorigenic properties both *in vitro* and *in vivo*. These observations, though still at an early stage, suggest that GRP78 could have a critical role in regulating CIC proliferation and survival, as well as in stem cell biology in general, as evidenced by the previous observation that homozygous knockout of *Grp78* led to massive apoptosis of cells within the inner cell mass, which are precursors of embryonic stem cells.<sup>46</sup>

### GRP78 IS AN EFFECTOR FOR LEUKEMOGENESIS AND AKT ONCOGENIC SIGNALING

Although GRP78 is established to protect cancer cells against the adverse hypoxic and nutrient-deprived microenvironment of solid tumors, its role in the initiation and progression of hematologic cancers is just emerging. Proteomic analysis reveals that GRP78 is differentially expressed in the HSC-like fractions from the BM of leukemic patients.<sup>61</sup> In addition to being a tumor suppressor, PTEN also contributes to the maintenance of the HSCs. Induced deletion of *Pten* in the hematopoietic system of postnatal mice exhausted normal HSCs and promoted exhaustive proliferation of leukomogenic stem cells, resulting in the development of myeloproliferative disorders and eventually leukemia.<sup>62,63</sup> These studies further showed that the mTOR inhibitor rapamycin effectively suppressed growth of the leukemia-initiating cells and prevented the exhaustion of normal HSCs. Through creation of a biallelic conditional knockout mouse model of GRP78 and PTEN in the hematopoietic system, it was demonstrated that GRP78 haploinsufficiency potentially suppresses leukemogenesis and AKT/mTOR signaling in PTEN-null BM cells.<sup>23</sup> Importantly, *Grp78* heterozygosity by itself has no apparent effect on development or survival, nor does it alter total BM cell number or HSC population.<sup>23,46,47</sup> Thus, partial GRP78 expression is sufficient to maintain normal organ homeostasis whereas tumor progression requires an optimal level of GRP78, both in solid and blood tumors.

How might GRP78 contribute to the PTEN-null mediated leukemogenesis? Although GRP78 is able to confer multiple anti-apoptotic effects on cancer cells, a key mechanistic explanation may be that the activation of AKT, a prominent effector that is

activated by the loss of PTEN, is compromised by the reduction of GRP78 in the hematopoietic system. Thus, PTEN-null mediated AKT/mTOR signaling is potentially suppressed in the BM of the *Pten*-null *Grp78* heterozygous mice. It has been reported that ligation of cell surface GRP78 in human cancer cells with antibodies directed against its carboxyl domain suppresses PI3K/AKT/mTOR signaling.<sup>64</sup> The observation that knockdown of GRP78 in leukemia cells suppresses serum-stimulated phosphorylation of the p85 regulatory subunit of PI3K and inhibits PI(3,4,5) P3 production further suggests that GRP78 regulates AKT activation through functional regulation of PI3K.<sup>23</sup> Upregulation of Wnt signaling is also suggested to be associated with leukemogenesis.<sup>65</sup> Hypoxia-induced ER stress was reported to inhibit normal Wnt protein processing and secretion as ER stress causes dissociation between GRP78 and Wnt, which is essential for its correct posttranslational processing.<sup>65</sup> Hence, the delayed onset of leukemogenesis observed in the *Pten*-null, *Grp78* heterozygous mice may be partially attributed to the attenuation of Wnt protein processing due to GRP78 knockdown, which awaits further investigation. The expression of anti-apoptotic protein BCL-2 has been implicated in hematological malignancies.<sup>66</sup> Considering that GRP78 level may influence the binding of pro-apoptotic BH-3 protein BIK to BCL-2<sup>41</sup>, one speculation is that the suppressed leukemic phenotype in the *Pten*-null, *Grp78* heterozygous mice may in part be due to modulation of the BCL-2 activity. Although the role of GRP78 in the development of human leukemia remains to be validated, emerging evidence shows elevated *Grp78* mRNA and protein expression in patient leukemic blasts compared with normal controls, notably in acute myeloid leukemia and in high grade B-lineage lymphoid malignancies.<sup>21–23,67</sup> With the *Grp78* knockout leukemic mouse model providing proof-of-principle that a partial reduction of GRP78 can arrest leukemogenesis while having no harmful effect on the hematopoietic system, targeting GRP78 may represent a novel therapeutic target against leukemia and other stem cell related diseases.

#### GRP78 IS REQUIRED FOR ANGIOGENESIS IN THE TUMOR MICROENVIRONMENT

The tumor microenvironment contains a plethora of cells that support tumor growth and progression. Tumor vasculature is essential for tumor growth and metastasis, as it supplies the nutrients and oxygen that are critical for the growth and maintenance of the tumor. Endothelial cells are therefore the requisite members of the tumor microenvironment. The first hint that GRP78 may have a critical role in tumor angiogenesis is the constitutive high-level expression of GRP78 within the tumor vasculature of glioblastoma, suggestive of the constant state of stress of these activated tumor-associated endothelial cells.<sup>68</sup> Multiple lines of evidence support the critical requirement of GRP78 for tumor neoangiogenesis. In the *MMTV-PyVT* transgene-induced mammary tumor model, *Grp78* heterozygosity showed a dramatic reduction in the microvessel density of endogenous mammary tumors, whereas having no effect on the microvessel density of normal organs.<sup>47</sup> Wild-type syngeneic mammary tumor cells injected into *Grp78* heterozygous host mice showed suppressed tumor growth and the early phase of angiogenesis. As neoangiogenesis is critical for supporting metastatic growth, injection of WT, syngeneic melanoma cells in the *Grp78* heterozygous host mice also resulted in potent suppression of pulmonary metastatic lesions.<sup>69</sup> Furthermore, creation of a conditional heterozygous deletion of *Grp78* in the host endothelial cells showed a severe reduction of tumor angiogenesis and metastatic growth, again with minimal effect on normal tissue microvessel density.<sup>69</sup> Knockdown of GRP78 expression by siRNA in immortalized human endothelial cells revealed that GRP78 regulates endothelial cell proliferation, survival and migration.<sup>69</sup> Although intracellular GRP78 can

contribute to tumor angiogenesis, other forms of GRP78 may also be important. Cell surface GRP78 is induced by VEGF and is required for its angiogenic signaling.<sup>70</sup> Interestingly, some tumor cell lines are capable of secreting a high amount of GRP78 into the tumor microenvironment. By binding to the cell surface receptors of endothelial cells, extracellular GRP78 activates the ERK and AKT pathways, and protects endothelial cells from anti-angiogenic effects of bortezomib.<sup>35</sup> Angiogenesis inhibitors targeting VEGF and other pro-angiogenic growth factor pathways, while showing antitumor effects, concomitantly elicit tumor adaptations leading to accelerated metastasis.<sup>71,72</sup> Considering that GRP78 regulates a much larger repertoire of cellular function than a single pro-angiogenic pathway and is a key modulator of cellular adaptation to stress, targeting GRP78 may offer a new approach to dually suppress tumor growth and angiogenesis. The role of GRP78 in the microenvironment may well extend beyond the endothelial cells. The observation that GRP78 expression is upregulated in tumor-associated macrophages but not in normal organs<sup>73</sup> suggests that stromal cells supporting tumor growth are likely to experience stress associated with the tumor microenvironment and require GRP78 for proliferation, survival and migration. Thus, suppression of the stress induction of GRP78 may then have the added benefit of also impeding the ability of tumor-associated macrophages and other stromal cells to support tumor growth and this warrants future investigation.

#### TARGETING GRP78 SENSITIZES CANCER CELLS TO THERAPY

Therapeutic resistance remains a major challenge in the treatment of cancer. Although this issue is complex and involves multiple mechanisms, the induction of the protective elements of the UPR, such as GRP78, in both the tumor and the tumor microenvironment, particularly at the necrotic borders that are highly chemoresistant, can be a major contributing factor.<sup>18,32,73</sup> In agreement, an elevated expression of GRP78 correlates with resistance to a wide range of therapies including chemotoxic, anti-hormonal, DNA damaging and anti-angiogenesis agents in a variety of cancers, in both proliferating and dormant cancer cells as well as tumor-associated endothelial cells and inversely, knockdown of GRP78 sensitizes the cells to these treatments.<sup>27,38,39,68,74</sup> As the role of intracellular and cell surface GRP78 in cancer therapy and drug resistance has been summarized in previous reviews,<sup>5,33</sup> here we focus on some new, unanticipated findings.

Histone deacetylase (HDAC) inhibitors represent a new class of anticancer compounds with great therapeutic potential through their ability to modulate the expression of both histone and non-histone genes resulting in growth arrest, increased differentiation and apoptosis.<sup>75</sup> However, this treatment could also lead to resistance that is not well understood. It was recently discovered that HDAC inhibitors specifically induce GRP78 without concomitantly inducing an ER or heat shock stress response.<sup>76</sup> This is because of HDAC1 binding to and acting as a repressor for the basal transcription of *Grp78* and HDAC inhibitors such as trichostatin A and MS-275 relieve this suppression. Overexpression of GRP78 confers resistance to HDAC inhibitor induced apoptosis, whereas knockdown of GRP78 sensitizes cancer cells to the treatment. This provides an example that drugs that do not induce ER stress can still upregulate GRP78 through mechanisms distinct from the UPR. Another interesting observation is that in the case of estrogen-receptor positive breast cancer cells, whereas estrogen starvation initially lowers GRP78 expression, prolonged exposure to this adverse condition, which mimics the action of aromatase inhibitors, leads to the upregulation of GRP78, associating with hormonal resistance, which can be suppressed by the knockdown of GRP78.<sup>41</sup> In prostate cancer, GRP78 upregulation associates with androgen receptor status and the development of castration resistance and recurrence.<sup>53,57</sup>



With regard to drug resistance in leukemia, PI3K/AKT is constitutively active in primary acute myeloid leukemia cells from patients and blocking PI3K with inhibitor (LY294002) potentiates the response to AraC.<sup>77</sup> GRP78 is required for PI3K/AKT signaling in leukemia cells and modulation of GRP78 expression alters sensitivity to AraC.<sup>23</sup> As knockdown of GRP78 by siRNA downregulates both intracellular and cell surface GRP78<sup>69</sup>, both forms of GRP78 could contribute to AraC resistance, with mechanisms involving cell surface GRP78 promoting AKT survival signaling and intracellular GRP78 suppressing caspase-7 activation by AraC.<sup>23</sup> Resistance of B-ALL cells to the anti-leukemic drug vincristine was suppressed by (–)-epigallocatechin gallate, which inhibits the anti-apoptotic function of GRP78 by targeting its ATP-binding domain.<sup>67,78</sup> Chemoresistant B-ALL cells underwent apoptosis when exposed to a doxorubicin-conjugated penetrating cyclic anti-GRP78 peptide that targets cell surface GRP78.<sup>67</sup> There is also new evidence linking GRP78 overexpression to early relapse in childhood ALL,<sup>23</sup> as well as GRP78 overexpression in relapsed ALL compared with initial diagnosis.<sup>67</sup> Furthermore, ER stress induces alternative splicing of the *Grp78* transcript, leading to the production of a cytosolic isoform of GRP78 (GRP78va) that also protects leukemic cells from ER stress-induced cell death.<sup>79</sup>

Collectively, these discoveries identify GRP78 as a novel therapeutic target against chemoresistance in cancer cells. In agreement, recent studies showed that therapies capable of reducing the level of GRP78 in cancer cells are effective in enhancing cancer cell death, sensitizing them to chemotherapy treatment and reducing xenograft roles.<sup>5,33,67,80–85</sup>

#### CELL SURFACE GRP78 IS A NOVEL ANTICANCER TARGET

Evidence is emerging that a subfraction of GRP78 can be localized on the cell surface of cancer cells as well as cells undergoing ER stress.<sup>36,48</sup> The preferential expression of GRP78 on the surface of tumor cells *in vivo* enables specific tumor targeting by peptidic GRP78 ligands linked to cytotoxic agents for anticancer therapy without harmful effects on normal organs.<sup>86–89</sup> This is consistent with a report that a phage display-derived human monoclonal antibody that recognizes GRP78 binds strongly to multiple types of cancer cells, but shows weak or no binding to the corresponding normal tissues.<sup>90</sup> However, it should be cautioned that studies on cell surface GRP78 expression performed in tissue culture show much variability among different cell lines.<sup>36</sup> This is probably in part because of the inherent differences among cell lines that have been maintained long term in tissue culture. In addition, the stressful conditions of tissue culture may artificially induce cell surface localization of GRP78 in non-transformed cells. Another possibility is rapid endocytosis of cell surface GRP78 making its detection by acute methods not being reliable. Thus far, *in vivo* studies consistently showed preferential expression of surface GRP78 in tumors compared with normal tissues. A human monoclonal IgM antibody (SAM6) that was isolated from a gastric cancer patient bound to an O-linked glycosylated form of cell surface GRP78 in malignant cells and induced intracellular lipids accumulation before apoptosis induction in cancer cells.<sup>91</sup> Currently, a Phase I melanoma study is being conducted to test the efficacy of this antibody. Furthermore, hypoxic/stressed endothelial cells express cell surface GRP78, which is induced by VEGF.<sup>70</sup> Taken together, these findings raise the exciting idea that agents against cell surface GRP78 can dually target tumor cells and tumor vasculature while sparing normal organs.

#### CELL SURFACE CRT AND IMMUNOGENIC CHEMOTHERAPY

CRT is an evolutionarily conserved 46 kDa ER luminal protein traditionally regarded as a  $\text{Ca}^{2+}$  homeostasis regulator and an ER chaperone.<sup>92–95</sup> Additionally, CRT regulates other cellular processes, including cell adhesion, MHC class I molecules

assembly and hormone-sensitive gene expression. Although the majority of CRT resides in the ER, evidence from various studies indicates that CRT is also found on the cell surface and can be secreted from cells under specific conditions.<sup>92,96</sup>

#### CELL SURFACE CRT INDUCES IMMUNOGENIC TUMOR CELL DEATH

CRT was demonstrated to be expressed on the surface of tumor cells treated with apoptotic inducing agents such as anthracyclines, oxaliplatin and ionizing irradiation.<sup>97,98</sup> Additionally, cell surface CRT (ecto-CRT) functions as an “eat me” signal that is recognized by dendritic cells (DC), and elicits DC-mediated phagocytosis of tumor cells.<sup>99</sup> Phagocytosis leads to increased tumor antigen cross-presentation, which activates cytotoxic T cells and triggers an antitumor immune response and immunogenic tumor cell death. In human cancer samples, ecto-CRT expression is enhanced in both solid tumors and hematological malignancies when compared with the corresponding normal tissues, which could possibly explain the increased susceptibility of tumors in immune-based cancer therapy compared with normal cells.<sup>100</sup> In acute myeloid leukemia patients, the presence of ecto-CRT on leukemia blast cells undergoing anthracycline treatment positively correlates with the cellular anticancer immune response.<sup>101</sup>

The recent discovery that CRT is presented on the cell surface offers a new anticancer therapeutic strategy based on ecto-CRT-mediated immunogenic chemotherapy.<sup>102</sup> For example, human colon cancer cells that were treated with anthracycline in culture, and then injected into mice, prevented the tumor formation induced by the administration of non-anthracycline-treated colon cancer cells. Furthermore, intratumoral injection of recombinant CRT combined with the chemotherapeutic agent Mitomycin C cured already established tumors in the same mouse model.<sup>102</sup> These observations were also confirmed in fibrosarcoma and mammary carcinoma cells. Importantly, this tumor immunity was specific against autologous tumors, as opposed to unrelated tumors.<sup>102</sup> Interestingly, the immunogenic tumor cell death induced by ecto-CRT appears to require both cell surface CRT and apoptotic signals. CRT exposure driven by ER stress restores the immunogenicity caused by cisplatin, an apoptosis inducer that fails to induce CRT exposure by itself.<sup>103</sup> To induce immunogenicity, ecto-CRT must present on the surface of apoptotic cells, as opposed to the surface of adjacent live cells. Recombinant CRT that was absorbed onto the surface of tumor cells could trigger immunogenic tumor cell death when substituted for endogenous CRT that has translocated to the cell surface,<sup>99,102</sup> demonstrating the direct requirement of cell surface CRT in triggering immunogenic tumor cell death. Collectively, such tumor specificity raises the possibility of personalized tumor vaccination where primary tumor cells from a patient could be treated with anthracyclines *ex vivo*, and subsequently injected back into the patient in order to both eradicate existing tumor cells and to induce long-term antitumor immunity.

#### CELL SURFACE CRT EXPOSURE REQUIRES PERK ACTIVATION AND APOPTOTIC EFFECTORS

The cell surface exposure of CRT in immunogenic cell death results from the translocation of endogenous CRT and is unrelated to the enhanced expression of CRT within the cell.<sup>104</sup> Upon anthracycline treatment, only CRT and Erp5 are detected on the surface of tumor cells whereas other ER chaperones, including GRP78, GRP94, PDI or CNX are not. Erp57 and CRT form a complex in the lumen of the ER, and translocate together to the cell surface.<sup>105,106</sup> The translocation process requires the activation of PERK, but not IRE1 $\alpha$  or ATF6. Blocking the function of PERK-elf2 $\alpha$  activation using PERK-specific shRNA or non-phosphorylatable elf2 $\alpha$ -S51A mutant abolished CRT translocation, whereas

inhibiting the function of GADD34 and PP1 complex, which is involved in eIF2 $\alpha$  dephosphorylation, enhanced the surface exposure of CRT.<sup>98,103,104,107</sup> However, ER stress alone does not induce CRT/ERp57 translocation; additional apoptotic effectors are required. Caspase-8 cleavage downstream of PERK-activated eIF2 $\alpha$  phosphorylation is required for the cleavage of an ER-sessile protein called BAP31. BAP31 is a BCL-2-binding protein and cleaved BAP31 binds to BCL-2.<sup>108</sup> This binding may compete with the binding of BCL-2 to BAX, and could potentially lead to the activation of BAX and BAK, which serve as the apoptotic effectors required for CRT/ERp57 translocation downstream of PERK.<sup>104</sup> The physical translocation of CRT/ERp57 to the cell surface can be blocked by the anterograde protein transport inhibitor brefeldin A, the actin skeleton inhibitor latrunculin B or the knockdown of SNAREs, suggesting a role for the ER-Golgi secretory pathway.<sup>104</sup> Nonetheless, the precise roles of BAP31, BAX and BAK, or how CRT/ERp57 translocates from the ER to the cell surface have not been well elucidated.

### THE ROLE OF GRP94 AND OTHER CHAPERONES IN ANTITUMOR VACCINATION

GRP94, also known as gp96, is a major glycoprotein in the ER with functions beyond protein folding and processing.<sup>4</sup> GRP94 has an important role in immune responses by facilitating MHC class I molecule-mediated antigen presentation, and by inducing the maturation and activation of various cells involved in innate and adapted immune responses, including macrophages, DCs, T cells and B cells.<sup>109–111</sup> Therefore, it might be expected that GRP94 could elicit an antitumor response. Indeed, several studies demonstrated that a vaccination of lethally irradiated cancer cells expressing various non-ER-retainable autologous GRP94 fusion proteins (devoid of the ER retention/retrieval signal KDEL) protected mice from primary tumor growth as well as metastasis.<sup>112–114</sup> One explanation for this effect could be that autologous tumor-derived secretory GRP94 stimulates the maturation of macrophages and DCs, enhances the antigen cross-presentation and amplifies the inflammatory signals.<sup>109,110</sup> As an alternative to autologous GRP94, a pooled GRP94 vaccine derived from different multiple myeloma cells was shown to be as effective as the autologous GRP94 vaccine when tested in mouse models.<sup>115</sup> Taken together, these studies demonstrate that the use of GRP94 as an antitumor vaccination can prevent cancer development. However, GRP94 vaccination in mice appears less promising in the treatment of established tumors,<sup>114,116,117</sup> and clinical trials across various types of tumors suggest that autologous tumor-derived GRP94 vaccination may have limited efficacy.<sup>118–121</sup>

Tumor-derived GRP78 is also capable of eliciting an antitumor response and inhibiting tumor growth in mouse models with established tumors as well as inducing an antitumor memory response in mice rechallenged with the tumor cells.<sup>122</sup> One explanation for this effect could be that KDEL-deleted secretory GRP78 is recognized by DCs and cross-presented with associated antigens by MHC class I molecules, leading to the activation of cytotoxic T cells. Additionally, heat shock proteins HSP70, HSP110 and GRP170 derived from tumors have been shown to function as antitumor vaccines.<sup>123–126</sup> Evidence suggests that vaccination with mixed heat shock proteins or heat shock proteins with other antigens may increase the antitumor effect. In a mouse model injected with sarcoma cells, tumor regression and long-term survival is shown in the mice treated with a mixed heat shock protein antitumor vaccine (GRP94, HSP60, HSP70 and HSP110).<sup>127</sup>

### THE ROLE OF PDI IN TUMORIGENESIS

PDI is a thiol-disulfide oxidoreductase that is recognized for catalyzing the formation of disulfide bonds in newly synthesized

proteins in the lumen of the ER.<sup>128–130</sup> PDI is also found in the cytosol, nucleus, on the cell surface, and can be secreted<sup>131</sup> (Figure 3). Studies on the role of PDI in cancer have demonstrated a pro-oncogenic, pro-survival function for PDI in cancer and therapeutic resistance. Similar to other ER chaperones, PDI upregulation in response to ER stress helps ameliorate misfolded proteins and stress-induced apoptosis.<sup>132,133</sup> Thus, inhibiting PDI activity sensitizes cells to stress-induced apoptosis. Treatment of melanoma cells with the PDI inhibitor bacitracin enhanced the stress response and apoptosis in the presence of chemotherapeutic drugs.<sup>134</sup> PDI is highly expressed in invasive glioma cells and the invasive edge of human glioblastomas. Furthermore, suppression of PDI activity with an anti-PDI antibody, or the specific PDI inhibitor bacitracin, reduced tumor cell migration and invasion *in vitro*.<sup>135</sup> Beyond these roles in tumor progression, PDI may also be important for tumor formation. A recent study demonstrated a novel role of PDI in acute myeloid leukemia tumorigenesis, in which PDI suppressed the expression of CEBPA by binding to the stem loop region of its mRNA, and therefore blocking normal neutrophil differentiation.<sup>136</sup> PDI has also been reported to induce antibody production and evoke a potent humoral immune response in GM-CSF secreting cancer cells.<sup>137</sup> Therefore, because PDI is expressed on the surface of various murine and human cancer cell lines, and has important roles in tumorigenesis, it presents a potential immune target which may enhance the efficacy of anticancer therapy.<sup>135,137</sup>

### THE ROLE OF PERK IN TUMORIGENESIS

PERK is an ER transmembrane serine/threonine protein kinase that is activated in response to ER stress.<sup>1,2</sup> Upon UPR activation, PERK phosphorylates its two major substrates: eIF2 $\alpha$  and nuclear factor-like 2 (Nrf2).<sup>138</sup> (Figure 1). The phosphorylation of eIF2 $\alpha$  acts to attenuate global protein translation, whereas at the same time enhancing the translation of select mRNAs, such as ATF4.<sup>139</sup> The phosphorylation of Nrf2 activates the expression of enzymes that are required for ROS quenching.<sup>140,141</sup>

PERK has a critical role during fetal development since the *Perk*<sup>-/-</sup> mice that survive are characterized by severe postnatal growth retardation, skeletal dysplasia and neonatal diabetes, all of which are also symptoms presented in the human Wolcott-Rallison syndrome.<sup>142</sup> PERK is required for osteoblast proliferation, differentiation and bone matrix collagen I secretion.<sup>143</sup> PERK is also required for pancreatic  $\beta$  cell development. Despite displaying a morphologically and functionally normal pancreas at birth, mice with global *Perk* knockout rapidly developed hyperglycemia and died.<sup>142,144,145</sup> It was demonstrated that  $\beta$  cell development occurs at E13.5–16.5, and impaired  $\beta$  cell proliferation and differentiation was observed in *Perk*<sup>-/-</sup> mice from day E13.5 to 18.5.<sup>145</sup> Interestingly, mice with  $\beta$  cell specific *Perk* knockout expressed postnatally are normo-glycemic and maintain glycemia throughout the lifespan.<sup>145</sup> Taken together, these studies suggest that the defect of *Perk*<sup>-/-</sup>  $\beta$  cell development at the embryonic stage is permanent such that when  $\beta$  cells begin to have an important role in regulating blood glucose, the PERK deficiency starts to exert its effect and eventually leads to the hyperglycemic and neonatal diabetic phenotypes.

### DUAL ROLE OF PERK IN TUMOR PROLIFERATION AND SURVIVAL

In the case of tumorigenesis and cancer progression, PERK exhibits both pro- and antitumor properties. PERK promoted tumor survival and angiogenesis under conditions of ER stress caused by hypoxia, as was demonstrated in a xenograft tumor model derived from K-Ras or Ki-RasV12 transformed *Perk*<sup>-/-</sup> mouse



embryonic fibroblasts.<sup>146,147</sup> Similar observations were made in a xenograft model using human colorectal carcinoma cells expressing a dominant-negative PERK.<sup>146</sup> In these models, PERK-deficient tumors showed increased apoptosis in hypoxic regions, were poorly vascularized, and these effects were attributed to the loss of p $\text{eIF}2\alpha$  and ATF4. The requirement of PERK in tumor angiogenesis was further confirmed using a mouse *Perk*<sup>-/-</sup> insulinoma model, in which *Perk*<sup>-/-</sup> tumors had lower vascularity compared to size-matched *Perk*<sup>+/+</sup> tumors.<sup>148</sup> PERK is not the only kinase that can phosphorylate  $\text{eIF}2\alpha$  and regulate cell survival. GCN2 is another  $\text{eIF}2\alpha$  kinase demonstrated to work in parallel to PERK to regulate  $\text{eIF}2\alpha$ -mediated cell survival under conditions of hypoxia and nutrient deprivation in immortalized mouse embryonic fibroblasts, human fibrosarcoma and colorectal adenocarcinoma cells.<sup>149–151</sup> Mechanisms by which  $\text{eIF}2\alpha$  phosphorylation promoted cell survival were attributed to cell-cycle G1 arrest, mediated by the downregulation of cyclin D1 and the induction of HIF-1 $\alpha$  and p21<sup>WAF1</sup><sup>149,150</sup> and autophagy, mediated by ATF4 and CHOP.<sup>152–154</sup> Moreover, it has been shown that the expression of asparagine synthetase, which is a downstream target of ATF4, partially restored growth in tumors with compromised ATF4 function.<sup>151</sup> Although phosphorylation of  $\text{eIF}2\alpha$  attenuates protein translation and protects cells from apoptosis under ER stress, under severe stress such as bortezomib treatment, the downstream pro-apoptotic factor CHOP was activated and the cancer cells underwent apoptosis.<sup>155</sup> Bortezomib-resistant multiple myeloma cells displayed attenuated  $\text{eIF}2\alpha$  phosphorylation and became susceptible when  $\text{eIF}2\alpha$  dephosphorylation was inhibited.

The role of PERK in cell proliferation has been controversial. In early studies, PERK- $\text{eIF}2\alpha$  was indicated to function as a barrier in the transformation of normal cells to cancer cells. Expression of a non-phosphorylatable  $\text{eIF}2\alpha$  mutant led to the transformation of mouse fibroblasts, and facilitated the transformation of human fibroblasts in conjunction with hTERT and the SV40 large T-antigen.<sup>156,157</sup> Non-tumorigenic mammary epithelial cells with a dominant-negative PERK or a kinase dead PERK mutant showed increased proliferation, displayed hyperplastic growth and developed into small tumor nodules after orthotopic implantation.<sup>158</sup> In human squamous carcinoma cells, pharmacologically induced PERK activation caused growth arrest *in vitro* and suppressed tumor growth *in vivo*.<sup>159</sup> This study implies that PERK activation in established tumors is sufficient to inhibit tumor proliferation. This suppression of tumor cell proliferation by PERK may be attributed to the  $\text{eIF}2\alpha$ -mediated inhibition of cyclin D1 synthesis.<sup>26,149,159</sup> However, other studies indicate that PERK is required for cancer cell proliferation. *Perk*<sup>-/-</sup> insulinoma induced by  $\beta$ -cell specific expression of SV40 large T-antigen displayed reduced proliferation compared with *Perk*<sup>+/+</sup> controls.<sup>148</sup> Cell-cycle arrest was observed upon PERK knock-down by shRNA in human breast carcinoma and esophageal carcinoma cells via reduced Nrf2 activity. The same study demonstrated that mammary gland-specific *Perk* deletion delayed tumor onset in MMTV-Neu breast cancer mice.<sup>160</sup> Phosphorylated Nrf2 activates the expression of enzymes required for ROS elimination. Therefore, reduced Nrf2 activity leads to ROS accumulation that causes cell-cycle arrest mediated by increased DNA damage. Collectively, these observations demonstrate the complexity and specificity of PERK in tumorigenesis and cancer progression, and illustrate that the role of PERK is dependent on its expression and activation, as well as cancer cell type, proliferation status, tumor microenvironment and the model system studied. Therefore, one can imagine the challenges involved in targeting PERK as an effective and generalized anticancer therapy. For instance, the suppression of tumor growth by either overactivation or depletion of PERK might also drive proliferative cancer cells into dormancy and thereby protect them from chemotherapy.

## THE ROLE OF PERK IN AUTOPHAGY

Autophagy can be activated upon ER stress as a defensive mechanism for maintaining ER homeostasis.<sup>10,16,161</sup> Depending on the context, autophagy either enhances cell survival by counterbalancing ER expansion or commits the cell to non-apoptotic death.<sup>162</sup> Studies have shown that autophagy alleviated ER stress-induced cell death in cancer cells while promoting cell death in non-transformed cells.<sup>10</sup> As two important mechanisms regulating ER homeostasis, the UPR and autophagy are coupled via the PERK- $\text{eIF}2\alpha$ -ATF4 pathway. The requirement of the PERK- $\text{eIF}2\alpha$ -ATF4 pathway in mediating autophagy is shown in different models, in which autophagy is induced by polyglutamine, radiation, ECM detachment and hypoxia.<sup>17,152–154,163</sup> In response to ER stress, PERK phosphorylates  $\text{eIF}2\alpha$  and activates its downstream transcription factor ATF4, which induces the expression of another transcription factor CHOP. Studies have shown that ATF4 and CHOP bind to the promoter of two genes essential for autophagy. ATF4 activates the transcription of *Map1lc3b*, whereas CHOP activates the transcription of *ATG5*.<sup>152</sup> MAP1LC3B and ATG5 are two essential proteins for autophagy. ATG5-dependent conversion of free LC3-I to membrane-bound LC3-II is an essential step for autophagosome formation,<sup>17</sup> whereas MAP1LC3B induction is necessary for replenishing the rapid degradation of MAP1LC3B during the prolonged autophagy.<sup>152</sup> Interestingly, ATF4 also induces autophagy in a PERK-independent manner. The induction of autophagy by Bortezomib treatment was dependent on the proteasome stabilization of ATF4 and the upregulation of LC3B by ATF4.<sup>164</sup> However, it was also suggested that the UPR and autophagy are linked by IRE1, rather than PERK. The accumulation of LC3-positive vesicles triggered by tunicamycin or thapsigargin was shown to depend on IRE1 but not PERK or ATF6, using mouse embryonic fibroblasts or embryonic stem cells deficient for IRE1 $\alpha$ , ATF6 or PERK.<sup>16</sup>

## THE ROLE OF IRE1-XBP1 AXIS IN TUMORIGENESIS

XBP1 is a transcription factor downstream of the IRE1 $\alpha$  endonuclease.<sup>165–167</sup> It has been shown to be essential for the development and function of various organs, especially secretory organs. *Xbp1* knockout mouse embryos died of liver failure and severe anemia. Mice with liver-specific overexpression of XBP1 were rescued from the embryonic lethality, but died shortly postnatal with impaired secretory apparatus including the pancreas and salivary glands.<sup>165</sup> *Xbp1* and its splicing are also essential for plasma cell differentiation and antibody secretion.<sup>7,168</sup>

*Xbp1* splicing is widely observed in both solid and hematological tumors, and enhanced *Xbp1* splicing has been associated with more malignant phenotypes and poor survival.<sup>169–171</sup> XBP1 activation was required for tumor survival in solid tumors that undergo hypoxic stress.<sup>172</sup> In breast cancer cells, XBP1 was shown to be a key factor affecting estrogen dependency. Overexpression of XBP1-s in estrogen-dependent breast cancer cell lines conferred an increased expression of estrogen receptors and estrogen-independent growth.<sup>173</sup> In the case of virus-associated cancers such as the Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma, XBP1 was shown to bind to the promoter of EBV oncoprotein LMP1 and activate its expression.<sup>174</sup> Other than promoting cancer cell survival and facilitating tumorigenesis along with other oncogenes, XBP1 splicing itself may lead to tumorigenesis, as the expression of XBP1-s alone in B cells and plasma cells drove the pathogenesis of multiple myeloma with features mimicking the classic hallmarks of human multiple myeloma.<sup>175</sup>

As XBP1 is the only known substrate of IRE1 $\alpha$ ,<sup>167</sup> studies on IRE1 $\alpha$  also shed light on the role of the IRE1 $\alpha$ -XBP1 pathway in tumorigenesis. Using prostate cancer cell lines with IRE1 $\alpha$  mutants

that are either kinase-defective or with drug-inducible RNase activity, IRE1 $\alpha$  was reported to promote cell proliferation *in vitro* by regulating the expression of cyclin A1 through *Xbp1* splicing.<sup>176</sup> In a xenograft model with glioma cells expressing a dominant-negative IRE1 $\alpha$  (dnIRE1 $\alpha$ ), dnIRE1 $\alpha$  expressing cancer cells had a lower proliferation rate *in vitro* and *in vivo*.<sup>177</sup> This xenograft model also revealed that IRE1 $\alpha$  was required for angiogenesis and functioned as a key switch between the angiogenesis and the invasion of glioma. Wild-type gliomas showed the angiogenic/massive phenotype whereas tumors expressing dnIRE1 $\alpha$  displayed the avascular/diffuse phenotype. Inhibition of IRE1 $\alpha$  function diminished the expression of potent pro-angiogenic factors, leading to a compensatory response in glioma cells to migrate and hijack host blood vessels in response to their poor angiogenesis ability. The requirement of IRE1 in tumor angiogenesis under hypoxia or glucose deprivation could be attributed to its role in regulating VEGF-A expression.<sup>178</sup> However, the link between the IRE1-XBP1 axis, angiogenesis and tumor growth can also occur in a VEGF-independent manner. It was shown that secretion of VEGF was not compromised when XBP1 was inhibited using an shRNA approach.<sup>172,179</sup> Additionally, IRE1 $\alpha$  was recently reported to regulate the survival/apoptosis switch in the UPR.<sup>180</sup> Under mild ER stress conditions, IRE1 $\alpha$  preferentially splices XBP1 to promote cell survival; whereas under conditions of overwhelming stress or chronic ER stress, it causes endonucleolytic decay of ER-localized mRNA, including those encoding ER chaperones such as GRP78, and renders apoptosis.<sup>180–182</sup>

The important role of the IRE1 $\alpha$ -XBP1 pathway in cancer progression presents a potential therapeutic target in anticancer therapy. Recently a small-molecule inhibitor targeting the endonuclease activity of IRE1 $\alpha$  was identified and tested in a multiple myeloma model.<sup>183</sup> This inhibitor blocked *Xbp1* splicing induced by ER stress both *in vitro* and *in vivo*. Treatment with this small molecule inhibited the growth of human multiple myeloma cells xenografted in host mice, and displayed cytotoxicity specifically on the multiple myeloma cells rather than normal blood cells freshly isolated from patients. Along with this small molecule inhibitor, another IRE1 $\alpha$  inhibitor that is salicylaldehyde-based was used in rhabdovirus mediated oncolytic viral therapy,<sup>184</sup> in which natural or genetically engineered replicating viruses selectively targeted tumor cells based on their altered genetic properties.<sup>185</sup> Blockage of the UPR by this IRE1 $\alpha$  inhibitor sensitized cancer cells to apoptosis in response to rhabdovirus infection and dramatically improved the oncolytic efficacy.<sup>184</sup>

## CONCLUSION

Although extensive cell culture studies have clearly implicated the contribution of ER chaperones and the UPR in cancer cell proliferation, survival and therapeutic resistance, the fruition of mouse models of cancer that allowed for the dissection of the physiological roles of various components of the UPR in relation to their effects on normal organ development and homeostasis validated their unique roles in tumorigenesis as well as in tumor angiogenesis. Evidence is also emerging that ER stress not only arms cancer cells with pro-survival adaptive pathways to combat the host surveillance mechanisms, it also promotes a vicious feedback cycle where cancer cells and stroma cells in the tumor microenvironment cross stimulate each other leading to tumor progression and metastasis. Knowledge of the involvement of ER stress and the UPR in the tumor microenvironment is at an early stage and offers exciting new opportunities for investigation. The discovery that major ER chaperones such as GRP78, GRP94, CRT and PDI all exhibit a cell surface form with activities distinct from those of the ER forms changes the paradigm of their function and expands their role in tumor progression and therapeutic resistance. Although ER stress can be exploited to enhance tumor

cell death, tumor cells are likely to acquire resistance to such therapy, in part by upregulating the pro-survival arms of the UPR such as the ER chaperones. With therapeutics targeting ER chaperones and UPR pathways currently in development, the next major breakthrough will come from directly testing whether these agents will be effective in reversing the course of cancer, either alone or in combination with existing therapy, and their impact on normal cell physiology.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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