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Down-regulation of PERK enhances resistance to ionizing radiation

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ABSTRACT

Although, ionizing radiation (IR) has been implicated to cause stress in endoplasmic reticulum (ER), how ER stress signaling and major ER stress sensors modulate cellular response to IR is unclear. Protein kinase RNA-like endoplasmic reticulum kinase (PERK) is an ER transmembrane protein which initiates unfolded protein response (UPR) or ER stress signaling when ER homeostasis is disturbed. Here, we report that down-regulation of PERK resulted in increased clonogenic survival, enhanced DNA repair and reduced apoptosis in irradiated cancer cells. Our study demonstrated that PERK has a role in sensitizing cancer cells to IR.

1. Introduction

Radiation therapy is an extensively used mode of treatment for a wide range of cancers. However, like many other anti-cancer agents, ionizing radiation (IR) induces stress response pathways [1–3]. Cancer cells have been known to utilise stress response mechanisms in order to evade death as well as to enhance survival [4,5]. Proteins targeted for secretion, along with membrane proteins, are synthesized and modified in the endoplasmic reticulum (ER). Apart from the expression of mutant proteins, changes in ATP, calcium and redox status that impede correct protein folding activates the unfolded protein response (UPR) or ER-stress signaling [6,7]. Initiation of UPR is mediated by PKR like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1) [6,7]. These three ER transmembrane proteins sense perturbations in ER and activate corresponding arm of UPR pathways. Though, activation of UPR is directed to resolve the disturbed ER homeostasis, it can lead to the activation of cell death pathways if the stress persists and damage is irreversible [6,7].

ER stress induces homodimerization and autophosphorylation of PERK and thereby its activation. Activated PERK phosphorylates eukaryotic initiation factor 2-alpha (eIF2α) and inhibits cap dependent translation of proteins to reduce further stress in ER [8]. Nevertheless, PERK mediated translational block is not absolute since this in turn activates the cap-independent translation of activating transcription factor 4 (ATF4). ATF4 aids in resolving ER-stress by inducing the transcription of ER chaperones and proteins involved in amino acid synthesis and transport [9]. However, in addition, ATF4 also induces the expression of C/EBP homologues protein (CHOP) [10,11], a transcriptional factor, which activates the expression of Bim, a pro-apoptotic member of Bcl-2 family [12] and represses the expression of anti-apoptotic protein, Bcl-2 [13]. Although, the activation of PERK is a part of survival UPR, its persistent activation is a switch to apoptosis via regulation of CHOP [14]. Interestingly, PERK has been previously implicated to play a significant role in the activation of autophagy in irradiated caspase-3/7 null cells [15]. However, it remains largely unknown whether PERK modulates cellular response to IR in cancer cells that harbour functional caspase-3. In this study, we examined the role of PERK in determining the sensitivity of human cancer cells that express functional caspase-3 to IR.

2. Materials and methods

2.1. Chemicals and antibodies

All chemicals were purchased from Sigma–Aldrich, Dorset, UK unless indicated otherwise. Rabbit polyclonal antibodies against PARP, Phospho-eIF2α, PERK, Grp78 / Bip and CHOP were obtained from Cell Signaling Technology, Danvers, MA. Rabbit polyclonal antibody against 53BP1 and Alexa Fluor 488-labelled anti-rabbit IgG secondary antibody were purchased from Novus Biologicals, Cambridge, UK and Molecular Probes, Paisley, UK respectively.

2.2. Cell culture and treatments

MDA-MB-231 cells and T98G cells were maintained in DMEM (PAA, Pasching, Austria) and EMEM (Lonza, Cambridge, UK),

Abbreviations: IR, ionizing radiation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DDR, DNA damage response; DDSBs, DNA double strand breaks.

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respectively. The medium was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Paisley, UK), 50 U/ml penicillin (Gibco) and 50 mg/ml streptomycin (Gibco) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. Irradiation was performed using 2 mm Cu filtered 225 kV X-ray source (XRAD225, Precision X-ray Inc. Branford, CT) at a dose rate of 1.71 Gy/min.

2.3. RNA interference

MDA-MB-231 or T98G cells (0.2×10^6) were transfected with PERK or scrambled siRNAs as described previously [16]. ON-TARGET SMART pool siRNA from Dharmacon (Thermo Scientific, IL) was used to silence the expression of PERK. The sequences used were following: CCA AUG GGA UAG UGAC GAA; GGU AGG AUC UGA UGA AUU U; GCA AUU AGC CUU AAG UUG U; AAA UUU GGC UGA AAG AUG A and scrambled siRNA sequences were AGC AGC ACG ACT TCT TCA AG. Scrambled siRNAs were obtained from Qiagen, West Sussex, UK.

2.4. Clonogenic cell survival assay

Survival fraction was determined by clonogenic cell survival assay as published [17].

2.5. Western blotting

Following experimental treatments, cells (0.3×10^6) were removed from the culture plates or flasks or dishes by scraping. The whole cell lysate preparation and Western blotting were carried out as previously reported [18]. Stripping of immunoblots for re-probing were carried out with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) according to manufacturer's protocol.

2.6. DNA damage analysis by immunofluorescence microscopy

Cells were plated on cell culture grade cover slip at a density of 0.05×10^6 cells per cover slip and allowed to adhere overnight before irradiation at room temperature (25 ± 2 °C). Following irradiation, the cells were fixed in methanol/acetone (1:1) at indicated time points. DNA damage was analyzed by using the immunofluorescence assay. Cells were permeabilised in 0.5% solution of Triton X-100 in phosphate buffered saline (PBS) and then blocked with a solution of 0.1% Triton X-100, 5% FBS and 2 mg/ml skimmed milk in PBS. After blocking, cells were incubated with anti-53BP1 rabbit antibody for 1 h at room temperature. Following primary antibody incubation, cells were washed with a 0.1% Triton X-100 in PBS washing buffer and incubated with Alexa Fluor 488-labeled anti-rabbit IgG secondary antibody for 1 h at room temperature. Cells were washed in PBS and counterstained with 4,6-diamidino-2-phenyindole (DAPI) containing mounting medium for fluorescent microscopy (Vectorshield, Peterborough, UK). Cover slips were mounted on microscopic slides and viewed using Zeiss Axiovert 200 M microscope (Carl Zeiss Micro Imaging, LLC, North America).

3. Results

3.1. Down-regulation of PERK results in increased resistance to IR

In order to understand the role of PERK in modulating cellular response to IR, its expression was down-regulated in human cancer cell lines MDA-MB-231 and T98G cells by using siRNA methodology. Both cell lines express functional caspase-3 [19,20]. Cell survival was determined by clonogenic cell survival assay at doses of 2, 4 and 6 Gy. Cells treated with PERK siRNA exhibited increased clonogenic cell survival compared to scrambled controls (Fig. 1A and Supplementary Fig. 1A). In PERK down-regulated MDA-MB-231 cells, there was a significant increase in cell survival at higher dose (Fig. 1A). Similarly, in T98G cells, silencing of PERK resulted in increased survival at higher doses (Supplementary Fig. 1A). Down-regulation of PERK was confirmed by Western blotting (Fig. 1B and Supplementary Fig. 1B).

Activation of UPR has been reported in irradiated cancer cells [2,3,15]. In order to examine whether IR activates PERK and subsequent phosphorylation of eIF2α, Western blotting of phospho-serine 51-eIF2α was performed. As shown in Fig. 1B and Supplementary Fig. 1B, increased phosphorylation at serine 51 of eIF2α was visible immediately after IR that was completely abrogated in PERK down-regulated cells. Taken together, the data suggested that IR induced the activation of the PERK–eIF2α pathway and silencing of PERK resulted in increased resistance to IR.

3.2. PERK-silenced MDA-MB-231 cells exhibit enhanced recovery from DNA damage

Upon DNA double strand breaks, p53 binding protein (53BP1) gets re-localized into discrete foci in a hierarchical manner along with other associated proteins to form ionizing radiation induced foci (IRIF) [21]. Induction and repair of DNA double strand breaks (DDSBs) were indirectly assessed by the appearance and disappearance of 53BP1 foci as reported previously [22,23]. Immunostaining and counting of 53BP1 foci showed discrete foci marking DDSBs which was dose dependent (Fig. 2A). As shown in the Fig. 2A and B, there was no change in the number of 53BP1 foci in PERK siRNA treated cells compared to scrambled controls immediately after IR. However, interestingly, following 12 h after IR, there was a significant reduction in the number of foci in

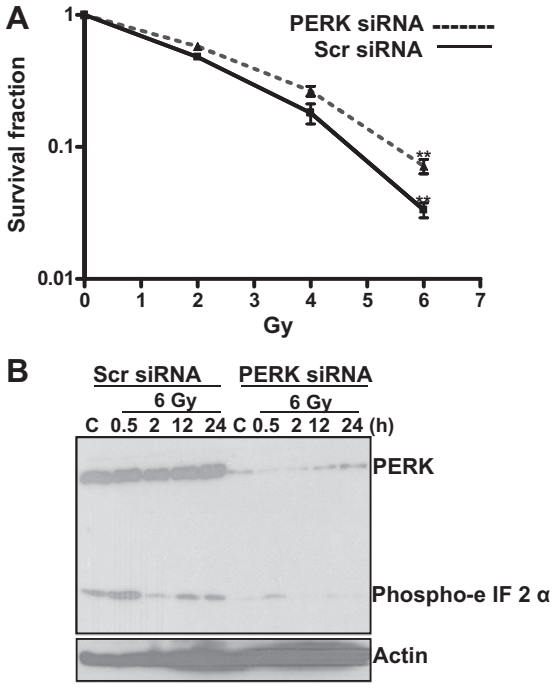

Fig. 1. Silencing of PERK increases clonogenic cell survival and inhibits phosphorylation of eIF2α in irradiated MDA-MB-231 cells. (A) Survival fraction was determined by clonogenic cell survival assay as described in Section 2. Survival fraction was normalized to corresponding un-irradiated controls. Data points show the mean of two independent experiments with triplicates ± SEM. $^{**}p < 0.001$. (B) Cells were lysed and the whole cell lysate (60 μg/well) were subjected to SDS–PAGE and immunoblots were performed with anti-PERK, anti-phospho eIF2α and Actin. C is un-irradiated control cells.

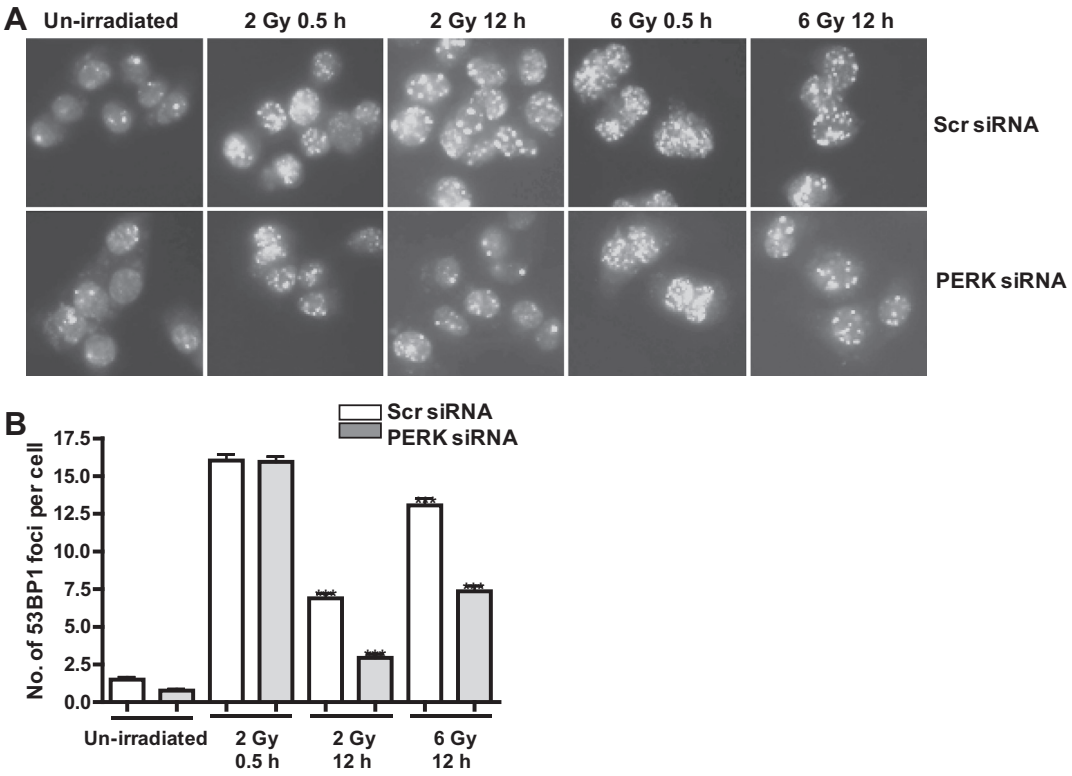

Fig. 2. Down regulation of PERK enhances DNA repair in MDA-MB-231 cells. (A) Immunofluorescence staining of 53BP1. (B) Number of 53BP1 foci was counted manually for fifty cells per slide for each sample and plotted. Data point shows the mean of two independent experiments ± SEM. $^{***}p < 0.0001$.

PERK-silenced cells (Fig. 2A and B). The disappearance of 53BP1 foci suggested that down-regulation of PERK has resulted in efficient and enhanced repair of damaged DNA. The data was also corroborated with the increased clonogenic cell survival exhibited by PERK-silenced MDA-MB-231 cells as shown in Fig. 1A.

3.3. Down-regulation of PERK attenuates apoptosis in irradiated MDA-MB-231 cells

ER stress has been implicated in radiation induced cell death [2,3,15]. Although, activation of PERK during ER-stress is pro-survival, persistent ER-stress initiate apoptosis via PERK–eIF2α–CHOP pathway [6,7]. In order to examine whether IR induces ER-stress in PERK-silenced cells, expression of canonical ER-stress markers was assessed. Grp78 or Bip is an ER-resident chaperone whose expression is up-regulated during ER-stress [6]. Western blot analysis of Grp78/Bip in irradiated cells transfected with scrambled siRNA showed no significant change in the expression (Fig. 3A). However, a slight increase in the expression of Grp78/Bip was observed in PERK down-regulated cells irradiated at both 2 Gy and 6 Gy (Fig. 3A). To examine whether IR activates the pro-apoptotic arm of ER-stress signalling in irradiated cells, immunoblotting of CHOP was performed. As shown in the Fig. 3A, CHOP was not induced in irradiated cells. CHOP is a pro-apoptotic transcriptional factor known to induce the expression of Bim that eventually lead to the intrinsic pathway of apoptosis [12]. On the other hand, cells treated with thapsigargin, a pharmacological inducer of ER-stress up-regulated the expression of both Grp78/Bip and CHOP (Fig. 3A). Interestingly, apoptosis was inhibited in irradiated cells treated with PERK siRNA as inferred by absence of the cleaved PARP (Fig. 3A). PARP is a substrate of caspase-3. Apoptosis in irradiated cells treated with scrambled siRNA was confirmed the appearance of cleaved PARP (Fig. 3A). Taken together, the data suggested that CHOP and Bip/Grp78 were not induced in the irradiated cells despite the activation of PERK–eIF2α pathway. To add further, IR activated apoptosis is PERK mediated but independent of CHOP. To examine the role of PERK–CHOP pathway in mediating ER-stress induced apoptosis, PERK-silenced cells were treated with thapsigargin. CHOP was not induced and ER-stress induced apoptosis was inhibited in PERK-silenced cells treated with thapsigargin (Fig. 3B). On the other hand, thapsigargin treated scrambled control cells that underwent apoptosis exhibited induced expression of CHOP (Fig. 3B). Activation of ER-stress induced apoptosis was monitored by Western blotting of PARP (Fig. 3B). This suggested that PERK dependent expression of CHOP is required for committing to apoptosis during the terminal phase of ER-stress as reported previously [24]. However, the data suggested that PERK regulates apoptosis in irradiated cells independent of CHOP.

4. Discussion

Activation of ER stress by IR has been reported in various cell types [2,3,15]. However, the relative impact of stress caused by IR is considered moderate compare to agents that severely affect ER-homeostasis [3]. In our study, it was apparent that IR does not cause an acute ER stress as there was no major induction of Grp78 compared to thapsigargin treated cells. However, PERK–eIF2α pathway was activated by IR. The absence of phosphorylated form of eIF2α in PERK-silenced cells suggested that transient block in protein translation in irradiated cells is indeed mediated by PERK. Even though, phosphorylation of eIF2α by PERK is meant to reduce the protein load during ER stress, it does modulate cell cycle progression and survival [25]. Specific and distinct activation of PERK arm of UPR has been reported in instances where expression of canonical ER stress markers remained unchanged [26]. The activation of PERK has been found to limit cell growth and ensue

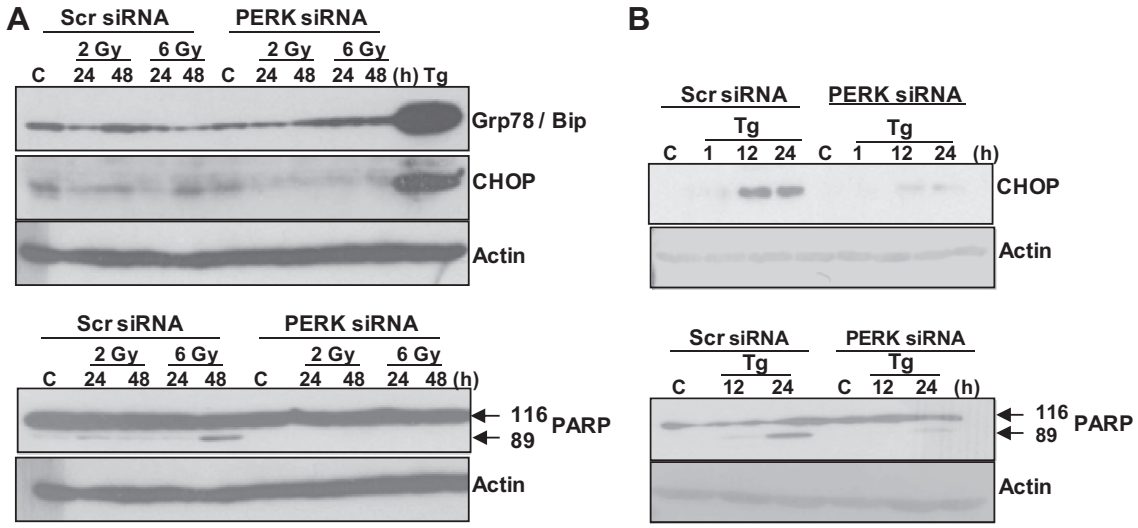

Fig. 3. IR induced apoptosis is CHOP independent and inhibited in PERK silenced MDA-MB-231 cells. (A and B) Immunoblotting was performed by using antibodies against Grp78/Bip, CHOP, PARP and Actin. Cells were treated with 0.5 μM thapsigargin (Tg) for 12 h in A and for indicated time points in B. C is un-irradiated/un-treated control cells.

acinar development [26]. Interestingly, we also observed that un-irradiated MDA-MB-231 cells silenced for PERK exhibited increased clonogenic cell survival (data not shown). Thus, it is plausible that PERK modulates cell cycle events critical in determining cell fate after irradiation.

Contradictory to the previous reports [2,3], IR does not activate significant ER stress in MDA-MB-231 cells. However, irradiation-induced apoptosis was abrogated in PERK-silenced MDA-MB-231 cells. Data from the available literature suggests the activation of the intrinsic pathway of apoptosis, which is often mediated by p53 or reactive oxygen species (ROS) in irradiated cells [27]. Defects in the apoptotic machinery activate alternate forms of cell death which is often characterized by autophagy. Activation of PERK–eIF2α pathway was found to be indispensable for IR induced autophagy in caspase-3/7 null cells [15]. However, interestingly, we demonstrated that silencing of PERK inhibited apoptosis in irradiated cells. Prolonged and unresolved stress in ER switches the adaptive ER-stress signaling to apoptosis. CHOP, a member of C/EBP transcriptional factors is a major determinant in activating apoptosis during this terminal phase of ER-stress [6]. CHOP is transcriptionally regulated by the PERK–eIF2α arm of ER-stress signalling machinery [11]. However, the expression of CHOP remained unchanged in irradiated MDA-MB-231 cells despite the activation of PERK–eIF2α pathway. This suggested that PERK dependent apoptosis in irradiated cells was not mediated by CHOP unlike in thapsigargin treated cells.

Enhanced recovery from DNA damage in PERK-silenced cells suggested efficient DNA repair mechanisms. However, PERK-silenced cells exhibited no difference from scrambled controls in terms of phospho-ATM levels (data not shown). In order to delineate the mechanisms behind the efficient DNA repair exhibited by PERK-silenced cells, the activation of other master regulators of DNA damage response (DDR) such as ATR and DNA-PKC has to be analyzed. The mechanistic details behind the resistance of PERK-silenced cells to IR are yet to be studied in detail. However, interestingly, it has been reported that PERK regulates the stability of cyclin D1 [25]. Cyclin D1 integrates growth and survival signals to the progression of cell cycle from G1 to S phase [28]. In addition, the over expression of cyclin D1 has been known to promote resistance to IR by enhancing the DNA damage response pathways [29]. Hence, the expression of cyclin D1 has to be determined in PERK-silenced cells after irradiation.

PERK has been paradoxically reported as both pro and anti-growth [4,5,26]. Tumour microenvironment is often overwhelmed with nutrient stress and hypoxia due to lack of a proper vasculature. Both of these conditions activate UPR and concurrently PERK [5]. ATF4, a downstream effector of PERK, aids cell survival by increasing the transcription of genes required for angiogenesis and survival [5]. Thus, evidently, PERK enhances tumorigenesis and required for cell survival in stressful conditions that exist in tumour environment. However, in contrast to the above reports, our study suggested a tumour suppressor role for PERK in irradiated cells. Anti-proliferative role for PERK is also evident from the reports where its enforced activation reduces tumorigenicity in various cell types [30,31]. In accordance with the previous reports [30,31], our study also proved that PERK activated upon stress limits cell proliferation and survival.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.129.

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