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Ribosomal protein L19 overexpression activates the unfolded protein response and sensitizes MCF7 breast cancer cells to endoplasmic reticulum stress-induced cell death

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ABSTRACT

Although first identified for their roles in protein synthesis, certain ribosomal proteins exert pleiotropic physiological functions in the cell. Ribosomal protein L19 is overexpressed in breast cancer cells by amplification and copy number variation. In this study, we examined the novel pro-apoptotic role of ribosomal protein L19 in the breast cancer cell line MCF7. Overexpression of RPL19 sensitized MCF7 cells to endoplasmic reticulum stress-induced cell death. RPL19 overexpression itself was not cytotoxic; however, cell death induction was enhanced when RPL19 overexpressing cells were incubated with endoplasmic reticulum stress-inducing agents, and this sensitizing effect was specific to MCF7 cells. Examination of the cell signaling pathways that mediate the unfolded protein response (UPR) revealed that overexpression of RPL19 induced pre-activation of the UPR, including phosphorylation of pERK-like ER kinase (PERK), phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), and activation of p38 MAPK-associated stress signaling. Our findings suggest that upregulation of RPL19 induces ER stress, resulting in increased sensitivity to ER stress and enhanced cell death in MCF7 breast cancer cells.

1. Introduction

Ribosomes are composed of a 40S small subunit and a 60S large subunit [1]. Each subunit is comprised of four ribosomal RNAs and approximately eighty different ribosomal proteins (RPs) with distinct functions [1]. The main constituent of the ribosome complex, RPs, are involved in the protein translational machinery of mammalian cells, and their functions are essential for cell growth, proliferation, and homeostasis [2]. Initially, RPs are synthesized in the nucleus and exported to the cytosol, where they function as chaperones to coordinate the interaction between ribosomes and RNA [3,4].

Although they are basically considered to be involved in protein production, some RPs are known to have pleiotropic functions that mediate a wide range of cell homeostasis-regulatory roles [5,6]. For example, RP-L5 can form a complex with mdm-2 and p53, implying that it plays a role in the regulation of p53 function [7]. Similarly, RP-S7 is a modulator of the p53-MDM2 interaction, implying that it has a tumor suppressor function [8]. RP-L11 was found to inhibit c-Myc activity and regulate c-Myc-associated ribosome biogenesis [9], and RP-S6 mediates cell death signaling induced by TRAIL in a signal-specific manner [10].

One RP that is predicted to have an extra-protein translational function is L19 (RPL19). RPL19 has been reported to play a role in prostate cancer physiology [11,12]. Global gene expression analysis of breast cancer specimens revealed that the RPL19 gene is amplified along with other genes mapped to chromosome 17q [13]. Various cancer-related genes have been characterized in chromosome 17, including the ERBB2 oncogene [13]. Although these previous reports supported the association of RPL19 with cancer cell physiology, the effect of gene amplification and protein overexpression of RPL19 in cancer cell death and survival was not characterized.

The unfolded protein response (UPR) is a cellular signaling pathway in the endoplasmic reticulum that is activated in response to overloading of unfolded or misfolded peptide chains. The UPR is composed of three phases (adapting, alarming, and cell death) with

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distinct signaling pathways active in each axis. Initially, the UPR functions as a protective mechanism to cope with changing environmental conditions. However, activation of the UPR also triggers stress-related signals that induce a pro-apoptotic cell death response [14]. Thus, activation of the UPR implies two possibilities. First, activation of the protective axis of the UPR implies that cells will overcome stressors that remain under the threshold level by changing the protein synthesis rate and lowering the peptide load in the ER. Second, however, prolonged or excessive levels of stress will eventually induce cell death with apoptotic characteristics [14].

In this report, we examined whether overexpression of RPL19 affects the sensitivity of breast cancer cells to cell death signaling. We found that overexpression of RPL19 activated the UPR in the absence of artificial ER stress induction and sensitized breast cancer cells to endoplasmic reticulum stress-induced cell death.

2. Materials and methods

2.1. Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM) was obtained from Thermo Scientific (Waltham, MA). Antibodies for eIF2α (Cat #SC-11386), p38 MAPK (SC-728), phospho-PERK (SC-32577), and PERK (SC-13073) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies for phospho-eIF2α (CS-3597), phospho-p38 MAPK (CS-9216), caspase-7 (CS-9494), and poly-ADP ribosyl polymerase (PARP, CS-9532) were purchased from Cell Signaling (Danvers, MA). Anti-α-tubulin antibody, thapsigargin, and tunicamycin were purchased from Sigma (St. Louis, MO). The antibody for CHOP was purchased from Thermo Scientific (Waltham, MA). Recombinant human TRAIL was purchased from Life Technologies (Carlsbad, CA).

2.2. Cell culture and transfection

All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 IU penicillin/streptomycin, and 1% L-glutamine (all from Life Technologies, Carlsbad, CA). For routine culture, cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transiently transfected with expression plasmids or small interfering RNAs using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Briefly, cells were seeded at a density of 5×10^5 cells/well in 6-well plates. After overnight incubation, cells were transfected with 1 μg of pcDNA 3.1 or pcDNA3.1-FLAG-RPL19 and incubated for an additional 16 h. Cells were subjected to cell death assays and western blotting analysis after incubation with ER stress-inducing agents for 2 and 6 h.

2.3. Immunoblotting

Cell lysates were prepared in cell lysis buffer (50 mM Tris–HCl, pH 7.4, 1% IGEPAL, 100 mM NaCl, 1 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1 mM β-glycerophosphate, and protease inhibitor) (Roche, Basel, Switzerland). Protein concentration was determined in the cleared lysates using a bicinchoninic acid assay. Whole cell lysates were denatured and resolved by 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After protein

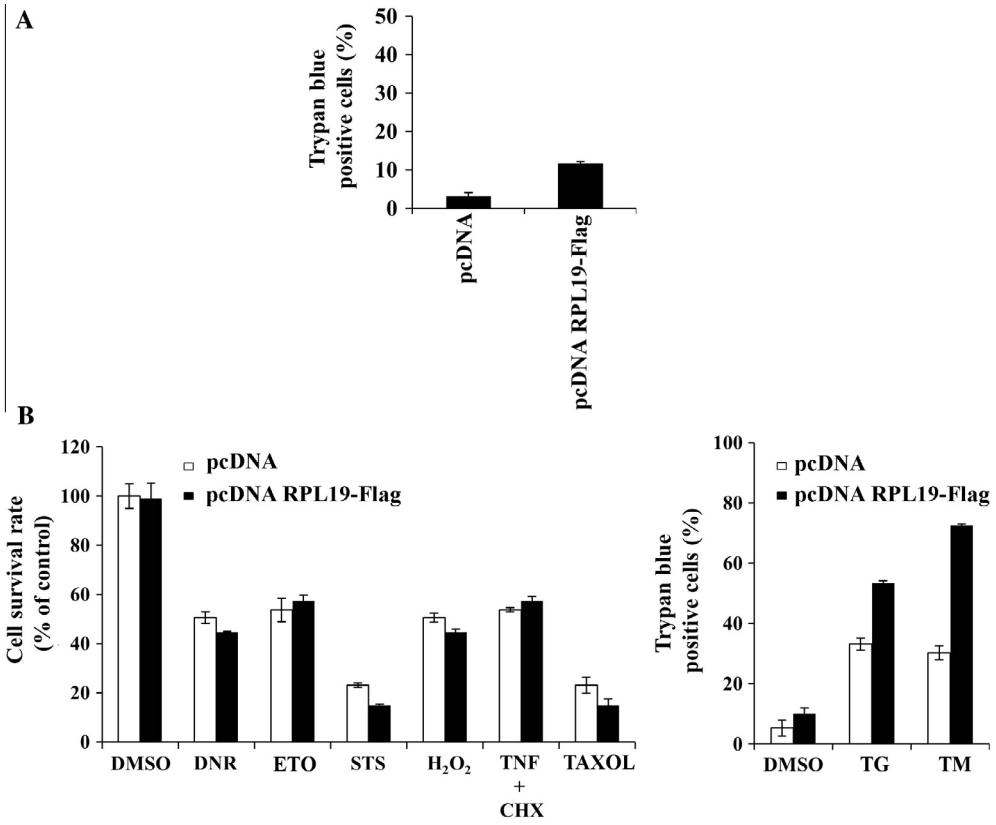

Fig. 1. Overexpression of RPL19 sensitizes MCF7 cells to ER stress-induced cell death. (A) Cytotoxic activity of RPL19 in MCF7 cells was analyzed. MCF7 cells were transfected with pcDNA empty vector or pcDNA-RPL19/Flag expression plasmids. After 24 h of transfection, cell death was evaluated by trypan blue exclusion assay. Data represent the mean ± SD ($n = 2$). (B) MCF7 cells with or without RPL19 overexpression were exposed to various cell death-inducing agents: 5 μM daunorubicin (DNR), 800 nM etoposide (ETO), 125 nM staurosporine (STS), 30 μM hydrogen peroxide (H_2O_2), 30 ng/ml tumor necrosis factor alpha with 10 μg/ml cycloheximide (TNF + CHX), 2.5 ng/ml paclitaxel (Taxol) 10 μg/ml tunicamycin, or 10 μM thapsigargin. Relative cell survival rates were evaluated by measuring cellular ATP contents or by trypan blue exclusion assay. Data represent the mean ± SD ($n = 2$).

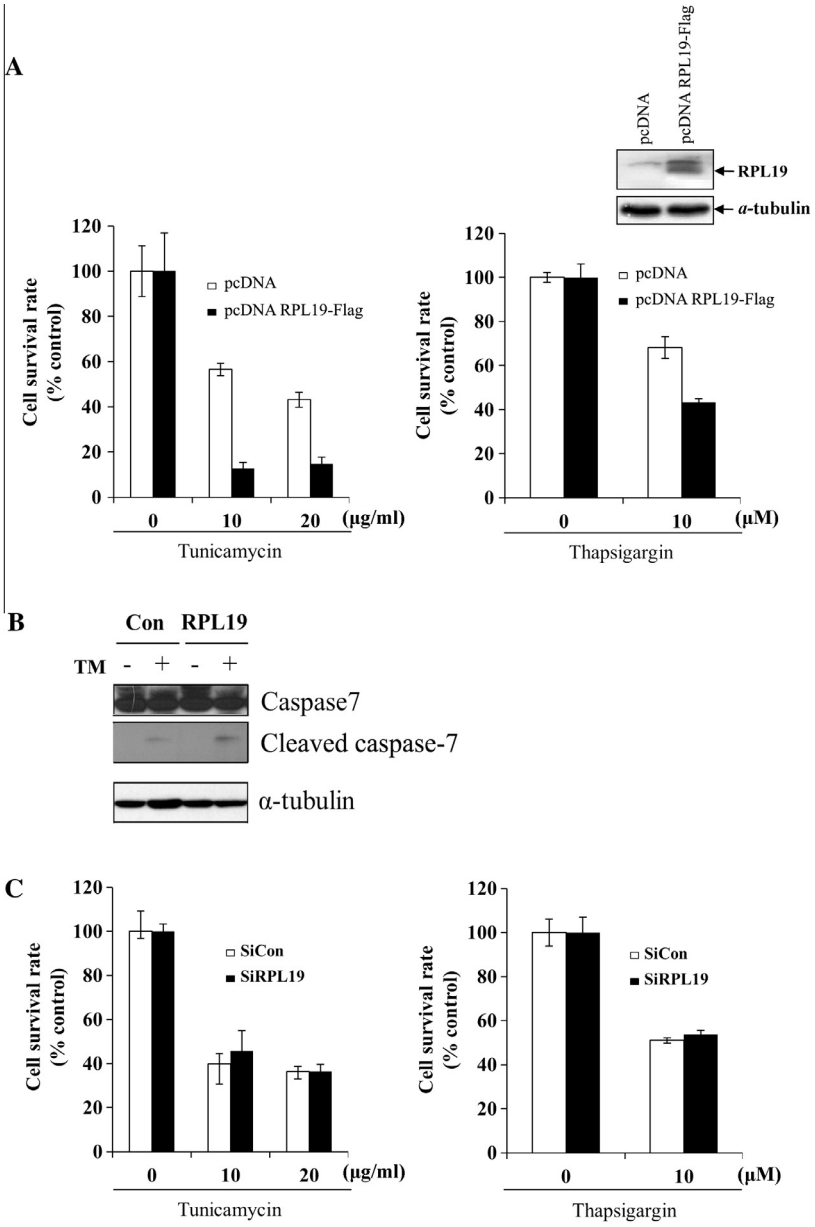

Fig. 2. Aberrant expression of RPL19 promotes cell death, but not cell survival. (A) MCF7 cells were transfected with pcDNA empty vector or pcDNA-RPL19/Flag expression plasmids. After exposure to ER stress-inducers with the indicated doses for 48 h, cellular ATP contents were evaluated and interpreted to determine relative survival. RPL19 overexpression was confirmed by western blotting using anti-FLAG antibody (inset). Data represent the mean ± SD ($n = 2$). (B) Western blotting analysis shows rapid activation of caspase-7 in RPL19-overexpressing MCF7 cells following ER stress induction. Data are a representative image of two independent experiments. (C) MCF7 cells were transfected with RPL19 small interfering RNA for 48 h and exposed to ER stress-inducing agents. Relative cell survival was calculated following the procedure in (A). Data represent the mean ± SD ($n = 2$).

transfer to polyvinylidene fluoride membranes (Immobilon P: Millipore, Billerica, MA), the blots were blocked in Tris buffered saline with 0.1% Tween-20 (TBST) containing 5% non-fat milk and incubated with each primary antibody (1:1000). Protein bands were visualized with a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate-Pierce, Rockford, IL). The membranes were stripped and reprobed with anti α-tubulin antibody to confirm equal loading.

2.4. Cell viability assays

Relative cell viability was evaluated by determining cellular ATP levels using a bioluminescence plate reader assay, and by calculating the ratio relative to control samples. Briefly, cells were seeded at a density of 10^5 cells/ml in 40 μL per well in 96-well polystyrene Lumitrac microtiter plates. The next day, cells were incubated with cell death-inducing reagents for the indicated times. At various times, 10 μl of assay solution was added, and raw luminescence was measured using a Victor-3 plate reader device (Perkin Elmer) in the luminescence mode. For the quality-based determination of cell death, the same samples were subjected to trypan blue exclusion assay.

3. Results

3.1. RPL19 overexpression sensitized MCF7 cells to ER stress-induced cell death

To investigate whether overexpression of RPL19 affects the cellular response to anti-cancer therapeutic reagents, we evaluated

cell viability with or without concomitant cell death induction. MCF7 cells were transfected with pcDNA3.1 or pcDNA3.1-RPL19, and cell death induction was assessed. Although a minor increase in cell death (3–5%) was observed, overexpression of RPL19 did not modulate cell viability to a statistically significant level (Fig. 1A). Next, we investigated whether overexpression of RPL19 could sensitize MCF7 cells to cell death-inducing agents. At 24 h after RPL19 transfection, cells were treated with daunorubicin (DNR, a doxorubicin analog that intercalates with DNA, thus inducing DNA damage), etoposide (ETO, an inhibitor of topoisomerase II), staurosporine (STS, a pan kinase inhibitor), hydrogen peroxide (H_2O_2, a reagent producing reactive oxygen radicals), tumor necrosis factor alpha with cycloheximide (TNF + CHX, which induces receptor-mediated cell death), paclitaxel (Taxol, a mitotic inhibitor), thapsigargin (a sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor that induces ER stress), and tunicamycin (an *n*-lined glycosylation inhibitor that induces ER stress). As shown in Fig. 1B, cells overexpressing RPL19 were more sensitive to ER stress-induced cell death. Overexpression of RPL19 increased ER stress-induced cell death by thapsigargin from 33% to 53% in response to thapsigargin, and from 30% to 72% in response to tunicamycin. However, RPL19 overexpression did not modulate cell death triggered by other cell death-inducing reagents. Although a marginal increase in cell death was observed in RPL19-overexpressing cells treated with hydrogen peroxide (44–51%), staurosporine (14–23%), and paclitaxel (14–22%), it was not statistically significant compared to the marginal level of cell death induced by RPL19 overexpression itself (Fig. 1A). These data suggest that RPL19 overexpression sensitizes breast cancer cells to ER stress-induced cell death in a signal-specific manner.

To clarify the role of RPL19 in modulating the response to ER stress, we compared cell death in the presence of RPL19 overexpression or in the absence of RPL19 expression (Fig. 2). First, MCF7 cells were transfected with empty vector or an RPL19-expressing vector and treated with thapsigargin or tunicamycin as indicated (Fig. 2A). As shown in Fig. 2A, RPL19 overexpression alone had no effect on cell survival. However, similar to the findings shown in Fig. 1A, overexpression of RPL19 sensitized MCF7 cells to ER stress-induced cell death (tunicamycin and thapsigargin). While cells transfected with the empty vector showed 55% and 45% cell survival following tunicamycin treatment (10 μg/ml and 20 μg/ml, respectively), RPL19-overexpressing cells exhibited less than 10% survival. Additionally, RPL19 overexpression increased thapsigargin (10 μM)-induced cell death in MCF7 cells from 35% to 61% (Fig. 2A, right). Biochemical analysis supports our observation of RPL19-induced sensitization of ER stress-induced cell death. When MCF7 cells were treated with tunicamycin, the activation of executioner caspase-7 was faster in RPL19 overexpressing MCF7 cells (Fig. 2B).

In the next step, we assessed induction of cell death after knockdown of RPL19. However, loss of RPL19 did not specifically modulate induction of cell death induced by tunicamycin or thapsigargin (Fig. 2C). Taken together, these data suggest that RPL19 is involved in pro-apoptotic cell death signaling triggered by ER stress.

3.2. Cell death sensitization effect of RPL19 is MCF7 cell-specific

Next, we compared induction of cell death in response to ER stress in different breast cancer cell lines. In addition to MCF7, cell death in response to ER stress-inducing agents was examined in two additional breast cancer cell lines: T47D and MDA-MB-231. Cells were transfected with RPL19-expressing plasmids and treated with the indicated doses of thapsigargin or tunicamycin. After incubation for 24 h, cell death rates were evaluated. As shown in Fig. 3, RPL19 overexpression did not modulate the level of ER stress-induced cell death in any of the other breast cancer cell lines. Although RPL19 overexpression alone induced greater than 30% cell death in T47D cells (Fig. 3), increased sensitivity to ER

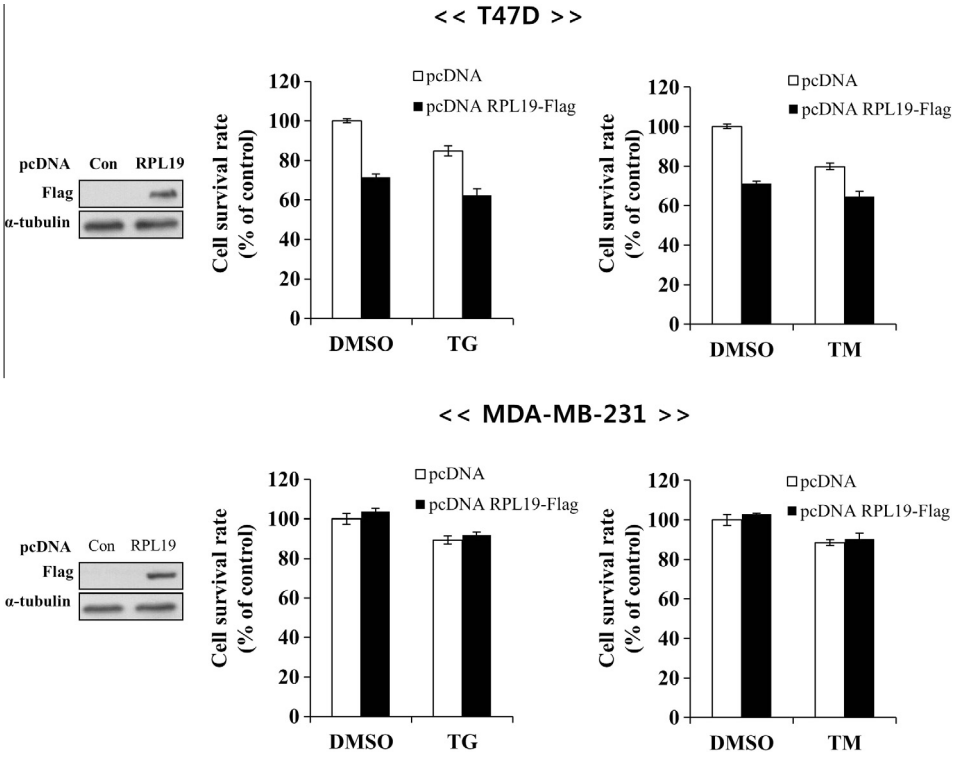

Fig. 3. Cell type-specific sensitization effect of RPL19 overexpression. Breast cancer cells, T47D (upper) and MDA-MB-231 (lower), were transfected with pcDNA empty vector or pcDNA-RPL19 expression plasmids. After 24 h of transfection, cells were exposed to ER stress-inducing agents for 24 h. Relative cell survival rates were evaluated by measuring cellular ATP contents. RPL19 overexpression was examined by western blotting. Data represent the mean ± SD ($n = 2$).

stress-inducing agents was not observed. These data suggest that the ER stress sensitization effect of RPL19 is cell-type specific.

3.3. RPL19 overexpression activates the unfolded protein response

Because RPL19-induced cell death sensitization was ER stress signal-specific (Fig. 1), we tested whether RPL19 overexpression affects UPR signaling in MCF7 cells. First, we overexpressed RPL19 in MCF7 cells and compared four marker proteins of the UPR. Changes in the expression level of GRP78, phosphorylation of PKR-like ER kinase (PERK), phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), and phosphorylation of p38 mitogen activated protein kinase (p38 MAPK) were examined. As shown in Fig. 4A, RPL19 overexpression did not modulate the expression level of GRP78, a marker protein of UPR activation. However, phosphorylation of PERK and its downstream signaling molecule eIF2α was increased. In addition, phosphorylation of p38 MAPK, a marker protein in the Ire1 axis of the UPR, was increased. These data imply that RPL19 overexpression activates UPR, even in the absence of artificial ER stress induction. We then

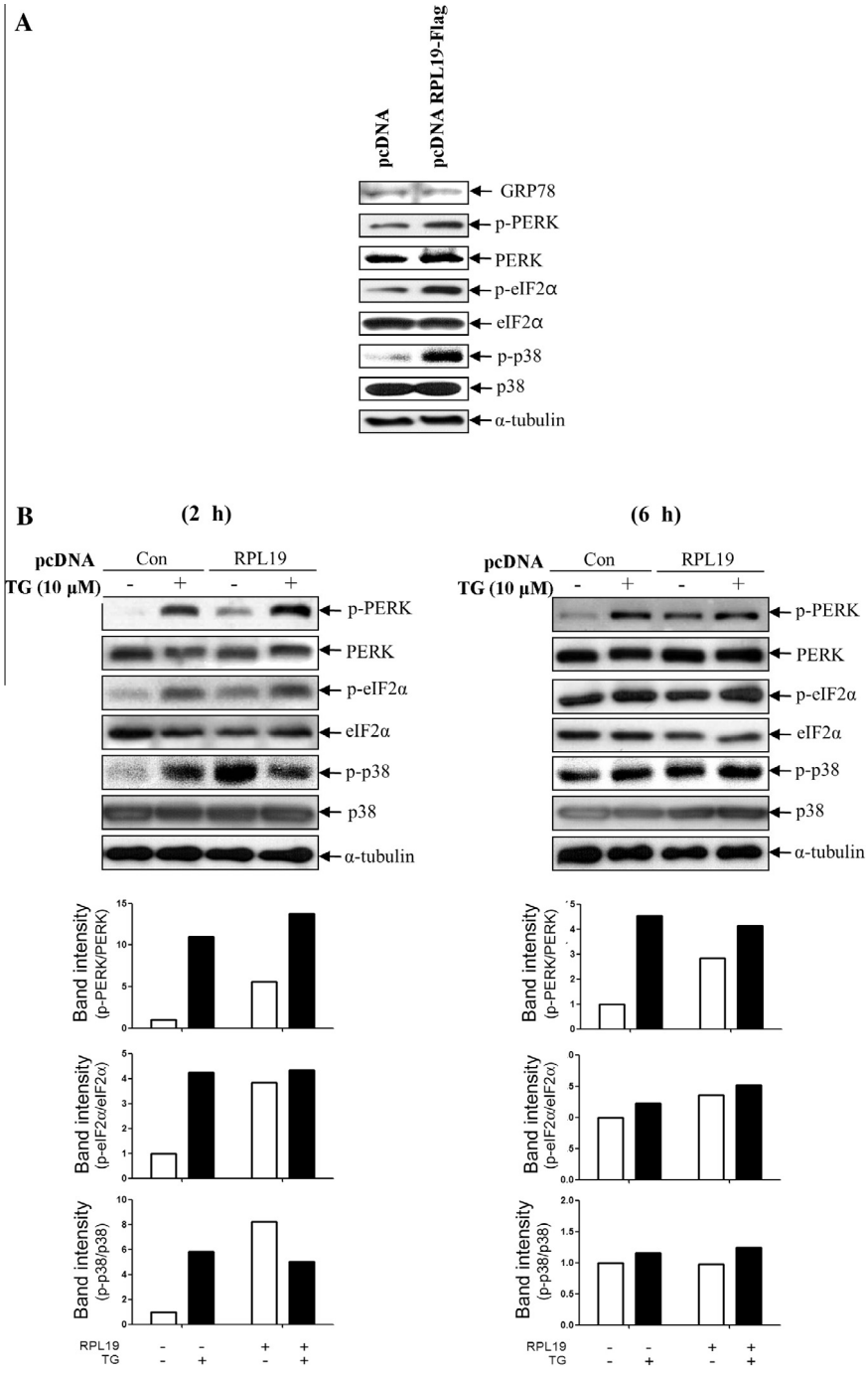

Fig. 4. RPL19 overexpression activates the unfolded protein response. (A) Cell extracts from MCF7 cells with or without RPL19 overexpression were prepared and subjected to western blotting analysis. (B) After transfection of RPL19 expression plasmids for 24 h, MCF7 cells were exposed to 10 μM thapsigargin for 2 h (left panel) or 6 h (right panel). Activity or expression of UPR proteins were analyzed by western blotting. A representative image of three independent experiments is shown. Band intensity of each target protein in western blotting analysis was evaluated and presented (lower graphs).

characterized the effect of RPL19 overexpression with or without ER stress induction. To clearly analyze UPR signaling, we compared UPR activation after ER stress induction at an early time point (2 h; Fig. 4B, left panel) and a late time point (6 h; Fig. 4B, right panel). At 2 h after ER stress induction, RPL19 overexpression induced phosphorylation of PERK, and accordingly phosphorylated eIF2α. In the Ire1 pathway, the alarming axis of the UPR, p38 MAPK was activated by RPL19 overexpression.

At 6 h of ER stress induction, PERK was still more phosphorylated. However, no differences in the phosphorylation of eIF2α and p38 MAPK were observed by RPL19 overexpression, implying that eIF2α and p38MAPK-associated UPR signaling was saturated and/or reversed.

Taken together, these data suggest that RPL19 overexpression triggers cell stress, which can activate UPR.

4. Discussion

In this report, we investigated our hypothesis that overexpression of ribosomal protein L19, which has been detected in breast cancers, can affect breast cancer cell survival. We examined whether RPL19 overexpression sensitized MCF7 cells to various cell death-inducing agents and found that overexpression of RPL19 sensitized MCF7 cells only to ER stress-inducing agents, including tunicamycin and thapsigargin. When treated by these ER stress-inducing agents, cell death rates were significantly increased compared with control cells, showing rapid activation of caspase 7 (Fig. 2B). However, knockdown of RPL19 did not modulate cell sensitivity to ER stress-induced cell death. In addition, sensitization to ER stress induced by RPL19 overexpression was observed only in MCF7 cells.

To determine the molecular mechanism by which RPL19 sensitizes cells to ER stress, we characterized the effect of RPL19 overexpression and found that it activated the UPR in the absence of artificial ER stress induction. The UPR pathways activated include PERK-eIF2α signaling and the stress-sensing Ire1 pathway. PERK, eIF2α, p38 MAPK were phosphorylated following RPL19 overexpression, implying that MCF7 cells overexpressing RPL19 were faced with an ER stress-like environment. However, expression of GRP78, a marker protein of UPR, was not upregulated under these conditions, implying that UPR activation did not simply result from the overloading of peptide chains, but rather as a result of more complex cell signaling.

Previously, it was demonstrated that RPL19 can act as a physiological regulator and prognostic marker of prostate cancer [11,12]. RPL19 mRNA overexpression was reported in malignant prostate cancer cells, and comparison of patient tissues with their benign counterparts showed 5–8-fold induction of RPL19 expression [11]. Kaplan –Meier analysis revealed that increased RPL19 expression is related to decrease prostate cancer patient survival, suggesting that RPL19 expression is a strong prognostic marker [11]. In a subsequent study, the role of RPL19 in promoting tumor formation was confirmed using transient and stable knockdown of RPL19 mRNA [12].

However, the notion of RPL19 as an oncogene does not entirely agree with our results. In our findings, decreased expression of RPL19 did not induce morphological changes in MCF7 cells (data not shown), nor did overexpression or knockdown affect the proliferation rate. Instead, RPL19 overexpression sensitized MCF7 cells only to ER stress-induced cell death in a cell type- and signal-specific manner. These results are in contrast to the common characteristics of oncogenic proteins, which are characterized by anti-apoptotic, pro-cell survival and enhanced cell proliferation activity.

The discrepancy and specificity of the cell death sensitizing effect induced by RPL19 suggests that the role of RPL19 in cancer progression and cell death signaling depends on the cancer and cell type. In addition, other RPL19-associated factors unique to MCF7 cells may be important in determining cell fate in response to ER stress. Future characterization of these factors will provide insight into the potential of treating breast cancers with ER stress-inducing anti-cancer agents.

We do not have a clear understanding of the mechanism by which RPL19 overexpression activates the UPR. Considering that GRP78 was not upregulated by RPL19 overexpression, activation of the UPR does not imply simple ER stress induction. Instead, it is possible that RPL19 can trigger unidentified pleiotropic stress signaling pathways, which in turn can activate the UPR without GRP78 induction. Prolonged stress signaling by RPL19 overexpression, although it does not induce cell death alone, may make the cells more responsive to cell death signaling.

Currently, ER stress-associated anti-cancer drugs are used only to treat multiple myeloma patients [15]. It is not clear whether ER stress-inducing agents can be used to treat breast cancers. Future characterization of the role of RPL19 overexpression and its cooperating partners in breast cancer cell death signaling will be a key factor for designing new modalities for the treatment of breast cancer by ER stress induction.

In conclusion, our data suggest a novel role for RPL19 in the induction of a pro-apoptotic stress response in cancer cells, and suggest a potential novel modality for the treatment of breast cancers.

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