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Phenylbutyric acid induces the cellular senescence through an Akt/p21^{WAF1} signaling pathway

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ABSTRACT

It has been well known that three sentinel proteins – PERK, ATF6 and IRE1 – initiate the unfolded protein response (UPR) in the presence of misfolded or unfolded proteins in the ER. Recent studies have demonstrated that upregulation of UPR in cancer cells is required to survive and proliferate. Here, we showed that long exposure to 4-phenylbutyric acid (PBA), a chemical chaperone that can reduce retention of unfolded and misfolded proteins in ER, induced cellular senescence in cancer cells such as MCF7 and HT1080. In addition, we found that treatment with PBA activates Akt, which results in p21^{WAF1} induction. Interestingly, the depletion of PERK but not ATF6 and IRE1 also induces cellular senescence, which was rescued by additional depletion of Akt. This suggests that Akt pathway is downstream of PERK in PBA induced cellular senescence. Taken together, these results show that PBA induces cellular senescence via activation of the Akt/p21^{WAF1} pathway by PERK inhibition.

1. Introduction

The endoplasmic reticulum (ER) is a major site not only for protein and lipid synthesis but also for cellular homeostasis regulation. After translocation of secreted or sorted proteins to the ER, folding process is monitored by quality control system in the lumen [1]. The accumulation of unfolded or misfolded proteins in the ER induces ER stress which activates the unfolded protein response (UPR) to halt protein translocation into the ER and increases the production of molecular chaperones. Prolonged ER stress resulted from protein aggregation leads UPR to induce apoptosis [2]. It was recently reported that various tumors are under high levels of ER stress and UPR is distinct from normal cells. Because of the rapid growth rate and glucose metabolism, signals generated by misfolded or unfolded proteins occur often in cancer cells [3]. Thus, it has been suggested that cancer cells utilize the UPR for survival during tumor growth.

ER stress responses can be initiated by activation of three different ER membrane-anchored proteins, namely PKR-like ER kinase (PERK) [4], inositol-requiring enzyme 1 (IRE1) [5,6] and activating transcription factor 6 (ATF6) [7]. Therefore, these proteins serve as sentinels for ER stress, which can threaten cell survival. The luminal domains of these three proteins are known to interact with Grp78/Bip, and accumulation of unfolded or misfolded proteins in the ER lumen is responsible for initiating UPR signaling, which decreases the interactions between the sentinel proteins and Grp78/Bip [8]. Activated PERK phosphorylates translational initiation factor eIF2α, resulting in reduction of global protein biosynthesis. Also, phosphorylated eIF2α induces specific mRNA translation, such as ATF4, which upregulates ER stress target genes [9]. ER-embedded ATF6 is separated from Grp78/Bip and translocates into the Golgi apparatus, where it is cleaved to form the nuclear ATF6. The nuclear form of ATF6 is involved in the induction of the UPR responsive gene by binding to the ER stress response element (ERSE) in the promoter [10]. Activated IRE1 functions as an endonuclease to cleave the mRNA of the X-box binding protein 1 (XBP1) into a form that encodes a functional bZIP transcription factor [11]. Subsequently, the spliced XBP1 protein upregulates several genes, including ER chaperones and enzymes, which provide relief during ER stress [12].

Generally, it has been thought that cellular senescence can occur through various upstream triggers, such as oncogenes and genotoxic reagents (Reactive oxygen species (ROS), UV, γ-irradiation, doxorubicine etc.) [13]. Although it is widely accepted that both p53/p21^{WAF1} and Rb/p16^{INK4a} pathways are major signaling pathways in cellular senescence [14], the precise mechanisms in ER stress induced senescence have not yet been determined [15]. However, recent evidence has suggested the possibility that ER stress is likely linked to the cellular senescence pathway. In

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addition, protein folding and the generation of ROS, as a protein oxidation product, occur in the endoplasmic reticulum [16,17]. $HRAS^{G12V}$-driven senescence in melanocyte is mediated by the ER-associated UPR [18,19]. Also, upregulation of ER stress has been shown to be responsible for oxidative stress induced chondrocyte senescence [20].

In this study, we identified a chemical chaperone, called PBA, that induces cellular senescence in some cancer cells, and demonstrated that the molecular mechanisms of this compound is related with the inactivation of PERK, which permits Akt/$p21^{WAF1}$ activation.

2. Results and discussion

2.1. Long exposure to PBA leads to cellular senescence in both MCF-7 and HT1080 cells

Because of the high proliferation rate, cancer cells require increased translation ability in comparison with normal cells. Moreover, cancer cells are more often exposed to severe conditions, such as hypoxia and nutrient starvation, during their lifetime, which results in the accumulation of unfolded or misfolded

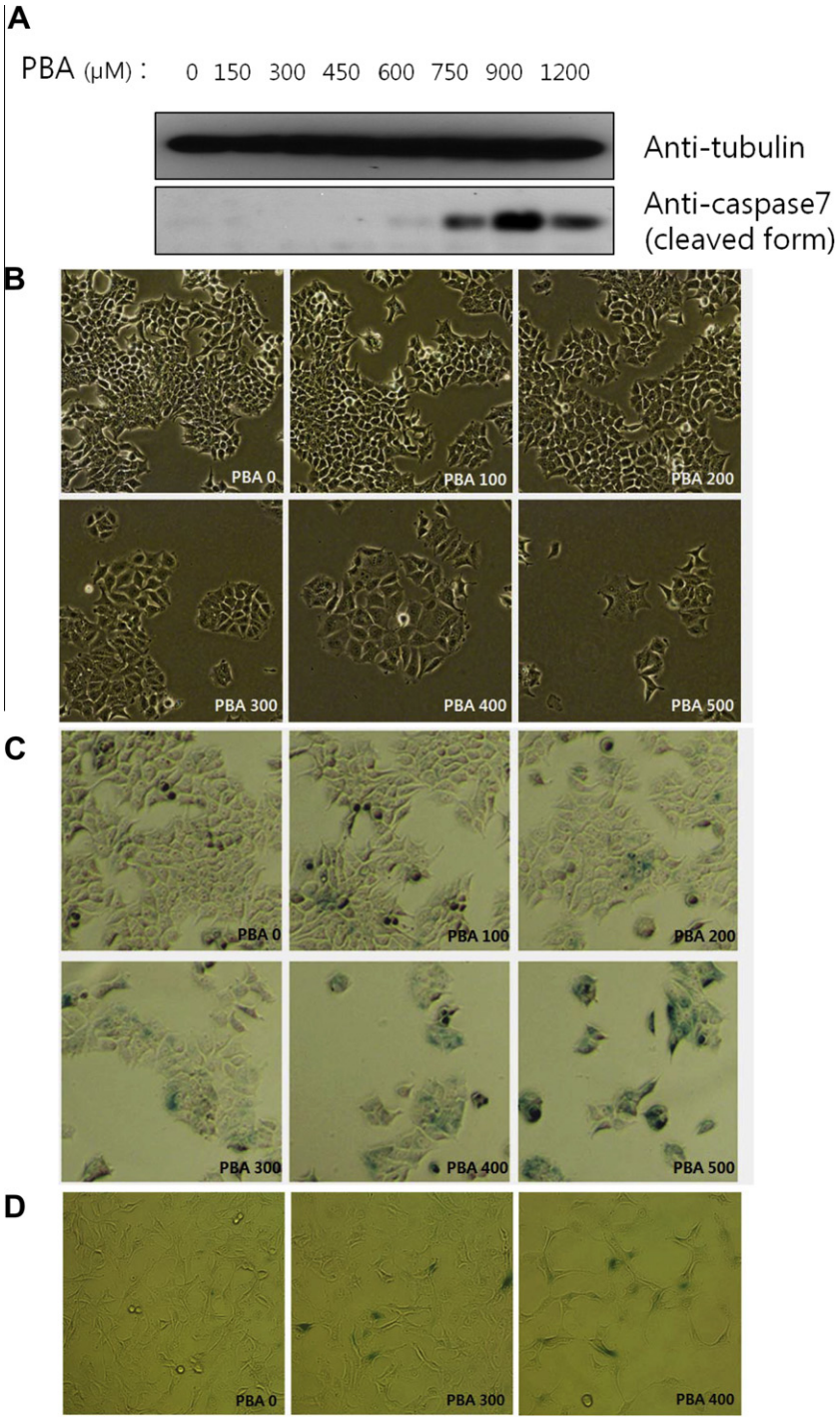

Fig. 1. PBA induces cellular senescence in both MCF-7 and HT1080 cells. (A) MCF-7 cells were treated with the indicated concentration of PBA for 3 days. The cell lysates were subjected to immunoblotting using the indicated antibodies. (B) MCF-7 cells were treated with the indicated concentration between 0 and 500 μM of PBA for 6 days. Cellular morphology observed under a light microscope. (C) The cells were subjected to SA-β-gal staining. (D) HT1080 cells were treated with 300 and 400 μM of PBA for 6 days, and SA-β-gal staining was performed.

proteins in the ER. Thus, to adapt to this condition, cancer cells seems to upregulate the UPR. Therefore, we hypothesized that a reduction of ER stress in cancer cells may facilitate the appearance of regressive phenotypes, such as apoptosis, cell cycle arrest and senescence.

First, to determine the optimal concentration of PBA which cannot induce apoptosis, MCF-7 cells were exposed to different concentrations of PBA for 3 days. According to previous reports [21], MCF-7 cells lack caspase-3 but induce the apoptosis pathway via caspase-7 activation [22]. Therefore, we evaluated the PBA-induced apoptosis by detecting the cleaved form of caspase-7. As shown in Fig. 1A, induction of apoptosis was evident when the PBA concentration was 750 μM. Next, we examined the cellular growth after exposing MCF-7 cells to PBA concentrations lower than 600 μM for 6 days (Fig. 1B). Interestingly, we observed a drastic morphological change in the MCF-7 cells treated with PBA at concentrations higher than 300 μM. These treated cells appeared flattened and enlarged (Fig. 1B). Since PBA-induced growth inhibition was not accompanied with apoptosis, the possibility that PBA-induced growth inhibition may result in cellular senescence was assessed. Also, an increase in senescence-associated-β-galactosidase (SA-β-gal) activity was also observed in these cells, which is a marker of senescence (Fig. 1C). To better understand the above finding, we next determined if PBA treatment had the same effect on HT1080 cells. PBA treated HT1080 cells also displayed an upregulation of SA-β-gal activity and a reduction in the cell number (Fig. 1D). Altogether, these data indicate that long exposure of PBA induces cellular senescence in both MCF-7 and HT1080 cells.

2.2. PBA activates Akt pathway, resulting in the upregulation of $p21^{WAF1}$

As a chemical chaperone, PBA is well known to improve the protein folding capacity of the ER. Also, as a histone deacetylase (HDAC) inhibitor [23], PBA is currently used as an anti-cancer drug. To confirm the action of this chemical chaperone, we examined the relationship between PBA treatment and ER stress in tunicamycin treated cells. Pretreatment of MCF-7 cells with 300 μM of PBA suppressed tunicamycin-induced C/EBP homologous protein (CHOP) and Grp78/Bip expression (Fig. 2A). Interestingly, a recent study showed that insulin receptor substrate (IRS), which is an upstream signaling molecule of both MAPKs and Akt/PKB, is activated in PBA treated mouse liver tissue [24]. To confirm this, we investigated various signaling molecules in cells treated with 100–300 μM PBA. As shown Fig. 2B, we only detected Akt activation, but neither JNK nor p38, in PBA treated MCF-7cells. Furthermore, we observed induction of $p21^{WAF1}$ and no effect on the level of p53 in a dose-dependent manner (Fig 2B). Therefore, these results suggest that the induction of $p21^{WAF1}$ is caused by the Akt pathway but not the p53-dependent pathway.

2.3. Inhibition of PERK but not ATF6 and IRE1 induces cellular senescence

Because treatment with PBA showed the same effect as overexpression of ER chaperones [25], we hypothesized that PBA-induced cellular senescence was linked to the inhibition of three sentinel proteins, ATF6, PERK and IRE1, which interact with Grp78/BiP in the ER lumen. Thus, we tested whether the knockdown of ATF6, PERK or IRE1 can induce cellular senescence in MCF-7 cells. As shown in Fig. 3A, PERK knockdown cells exhibited a significant induction of cellular senescence based on SA-β-gal activity when compared to control cells, while the ATF6 knockdown cells were slightly stained with SA-β-gal. In contrast, the cleaved form of caspase-7 was increased and the full-length PARP was reduced in IRE1 depleted cells, suggesting that IRE1 knockdown in MCF-7 cells elicited cell death (Fig. 3B). Next, we examined protein expression in PERK depleted cells. As expected, similar results were observed in the PERK depleted cells as those observed in the PBA treated MCF-7 cells (Fig. 3C). Knockdown of the respective genes was assessed 36 h after transfection (Fig. 3D). These data indicate that PBA induced cellular senescence was intimately connected with PERK inhibition. In addition, these results suggest that cancer cells may adapt to ER stress through UPR activation via differential expression of the sentinel proteins in ER.

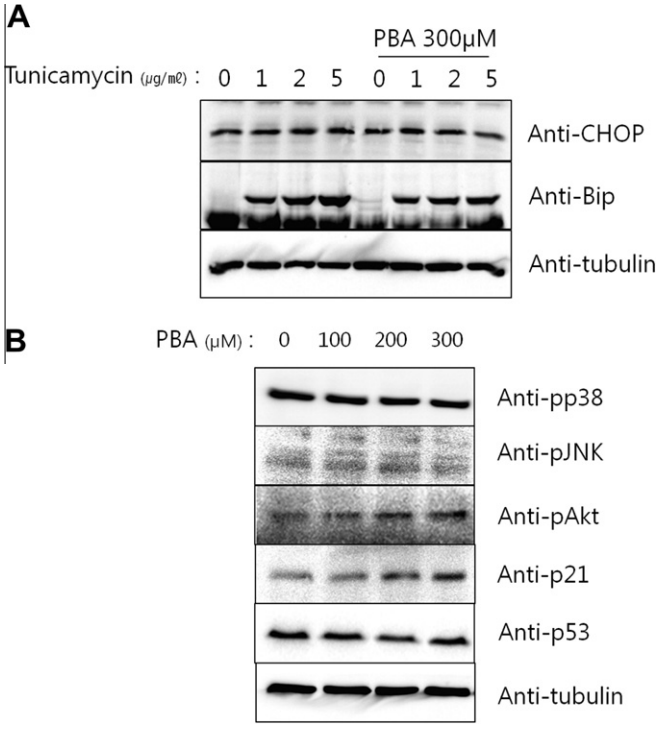

Fig. 2. PBA induces the activation of Akt, resulting in the upregulation of $p21^{WAF1}$. (A) MCF-7 cells were pre-incubated with or without 300 μM of PBA for 1 h and subsequently treated with various concentrations of tunicamycin. After 24 h, cell extracts were subjected to immunoblot analysis using the indicated antibodies. (B) MCF-7 cells were treated with the indicated concentration of PBA for 3 days. The cell lysates were subjected to immunoblotting with the indicated antibodies.

2.4. Akt/$p21^{WAF1}$ pathway activates in PERK depletion induced cellular senescence

To further verify that the cellular senescence induced by PERK depletion is related with Akt activation, we introduced Akt siRNA into MCF-7 cells in combination with PERK siRNA and measured SA-β-gal activity. As shown in Fig. 4A and B, the number of SA-β-gal positive cells in the Akt and PERK dpeleted MCF-7 cells was significantly lower than the PERK depleted cells. Because Akt is an upstream kinase that directly phosphorylates $p21^{WAF1}$ [26], we investigated the level of $p21^{WAF1}$ in the double depleted cells. As shown in Fig. 4C, $p21^{WAF1}$ induction was moderately reduced in the double depleted cells compared to PERK depleted cells. These results suggest that the cellular senescence induced by PERK depletion was closely connected with the activation of the Akt/$p21^{WAF1}$ pathway.

Cancer cells upregulate UPR to limit the effects of continuous ER stress [27], while the UPR pathways in most normal cells remain inactive. Indeed, the signaling molecules mediating the UPR pathway, such as XBP1, ATF6, CHOP and Grp78/Bip, have been reported to increase in breast tumors, gastric tumors, hepatocellular carcinomas, and esophageal adenocarcinomas [28]. This discrepancy

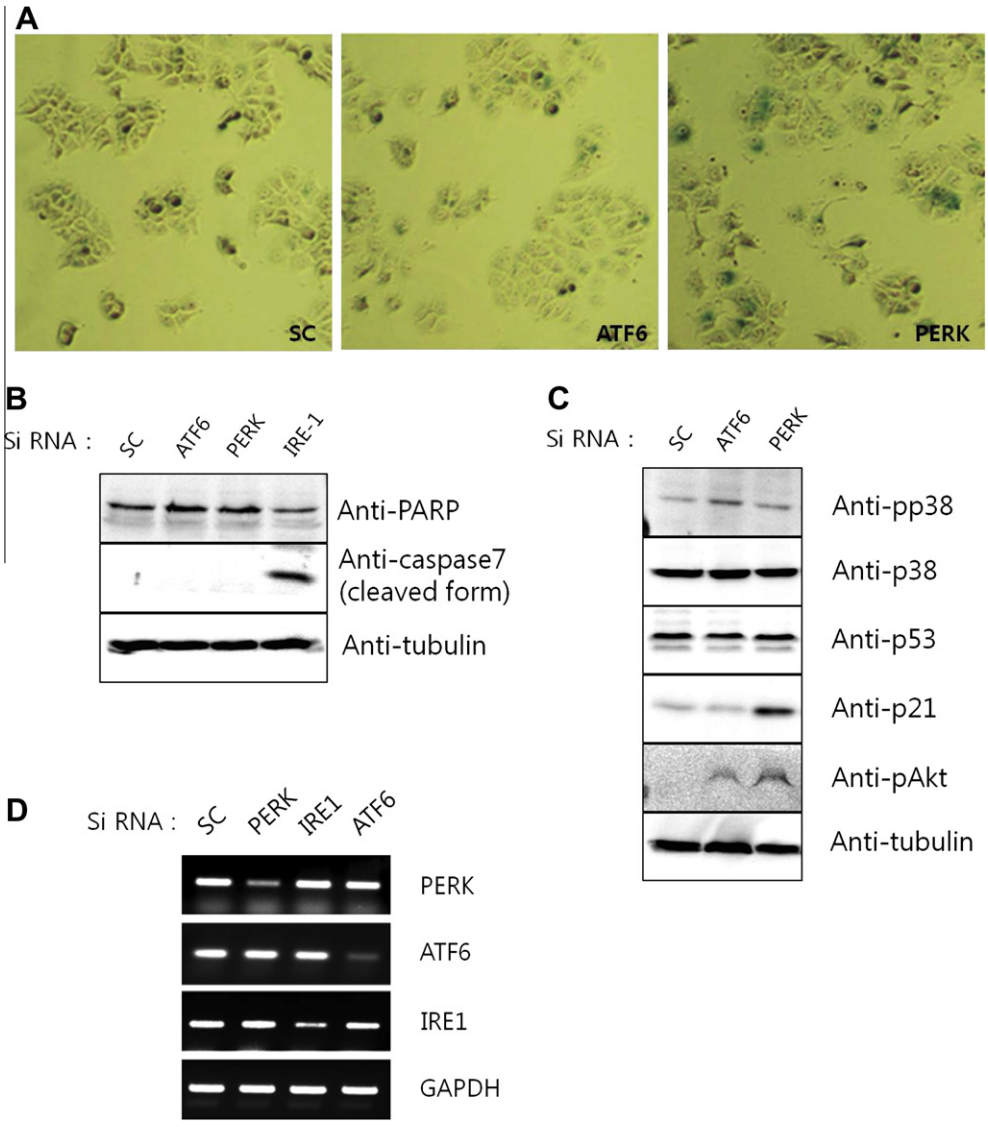

Fig. 3. The knockdown of PERK but not ATF6 and IRE1 also can induce cellular senescence. MCF-7 cells were transfected with control siRNA and siRNAs against and PERK, ATF6 and IRE1, respectively. (A) Seventy two hours after transfection, the cells were subjected to SA-β-gal staining. (B and C) The cell extracts were resolved using SDS–PAGE and subjected to immunoblot analysis using the indicated antibodies. (D) Each gene expression in MCF-7 cells was determined by RT-PCR analysis after treatment with control, PERK, ATF6 and IRE1 siRNAs, respectively.

between cancer cells and normal cells could be utilized for the development of drugs that target the UPR and achieve specificity in cancer therapy [29]. Therefore, the results presented in this study could be used to develop novel cancer therapies that target the UPR.

3. Materials and methods

3.1. Cell culture

Cells were cultured in RPMI-1640 (Human breast cancer MCF-7) or DMEM (Human fibrosarcoma HT1080), supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), penicillin (100 units/mL) and streptomycin (100 μg/ml) (Invitrogen, USA). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2.

3.2. Antibodies and reagents

Antibodies against $p21^{WAF1}$, p38MAPK, p53, CHOP, Bip, Akt and PARP were obtained from Santa Cruz Biotechnology (USA) and anti-α-tubulin was from Calbiochem (USA). Antibodies against pp38, pAkt, cleaved caspase-7 and pJNK were purchased from Cell Signaling Technology (USA) and anti-PERK antibody was from Abcam (United Kingdom). 4-Phenylbutyric acid (PBA) was purchased from Santa Cruz Biotechnology (USA) and tunicamycin was purchased from Sigma–Aldrich (USA). An enhanced chemiluminescence (ECL) system as a western blotting reagent was purchased from Roche (USA).

3.3. Immunoblotting and siRNA transfection

Cells were harvested and lysed on ice in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium dodecyl sulfate, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin). Cell extracts were quantitatively analyzed using the Bradford assay (Bio-Rad). Equal amounts of extract were resolved by 9–15% SDS–PAGE and subjected to immunoblot analysis. For siRNA transfection, specific siRNA oligos were purchased from Bioneer (Korea), SC (SN-1013), Akt (1004106), PERK (1009444), ATF6 (1171246), IRE1 (1046373), and were transfected

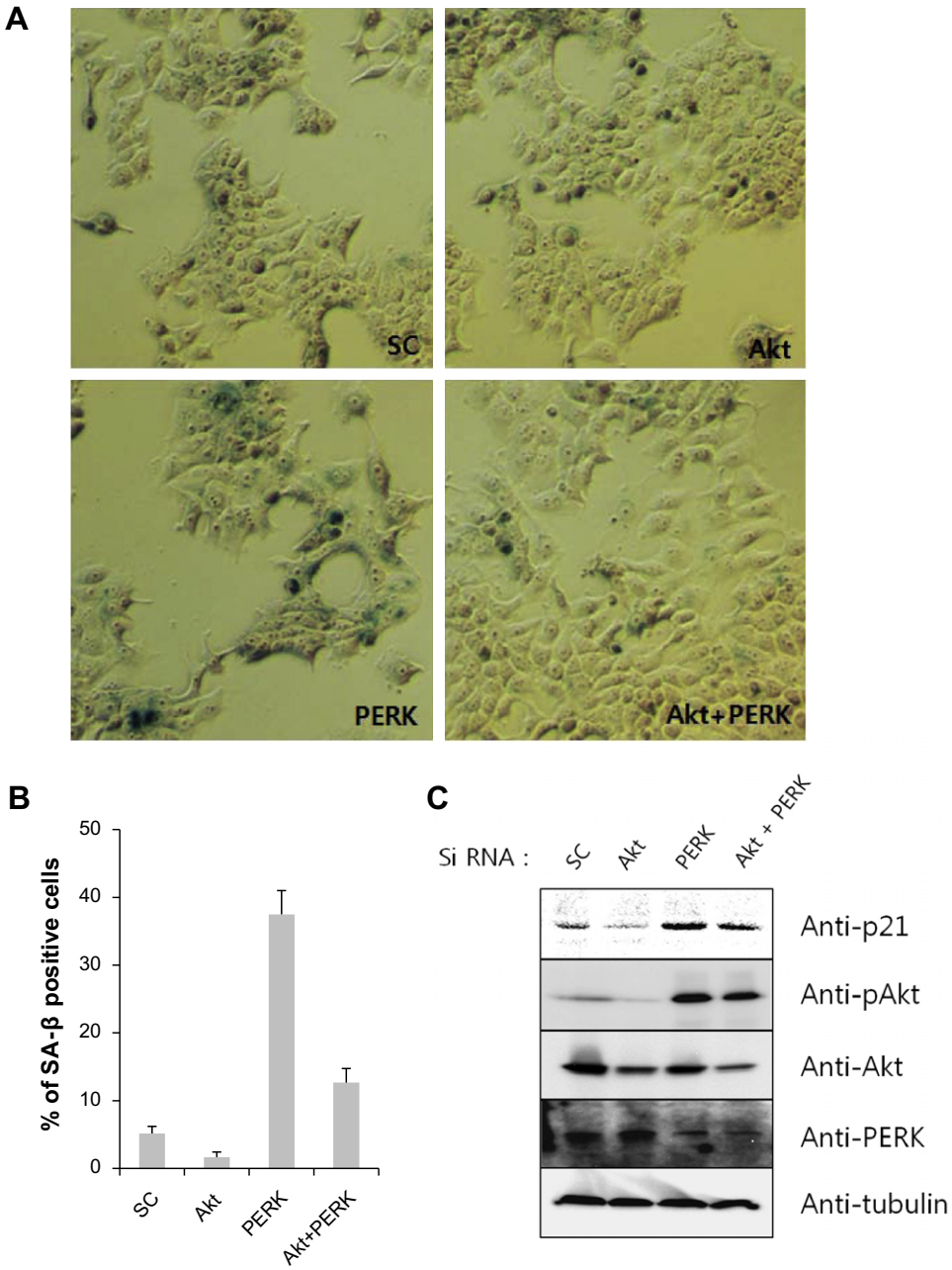

Fig. 4. PERK depletion induced cellular senescence is repressed by the inhibition of Akt. MCF-7 cells were transfected with control or PERK siRNA with or without Akt siRNA. (A and B) After 72 h, the cells were stained for SA-β-Gal and imaged under a light microscope. More than 200 cells in three different fields were counted, and the percentage of SA-β-gal-positive cells was plotted. (C) Twenty four hours after siRNAs transfection, the cells were harvested. Equal amounts of cell lysates were immunoblotted with the indicated antibodies.

using Lipofectamine RNAiMAX transfection reagent according to manufacturer's recommendations (Invitrogen, USA).

3.4. Senescence-associated β-galactosidase assay

Cells were fixed with 3% formaldehyde for 5 min at room temperature. After washing with PBS three times, the fixed cells were incubated at 37 °C with fresh SA-β-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase, 40 mM citric acid, 40 mM sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$) in the absence of CO_2. After staining for 12–16 h, positive cells for SA-β-gal activity were observed under a light microscope. More than 200 cells were counted in randomly chosen field from each sample.

3.5. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using the TRIZOL reagent. cDNA was synthesized from the total cellular RNA using oligo-dT primer (Promega, USA) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Gibco BRL, USA) at 37 °C for 1 h. The cDNA served as a template for PCR amplification of PERK, ATF6, IRE1 and GAPDH genes. The sequence of the PCR primers was as follows: PERK, 5′- TCA AAT GCA GCT GTG CAG AA -3′ and 5′- CTC ATC CTG GTC CAT TGC AG -3′; ATF6, 5′- GGA TTT GAT GCC TTG GGA GT -3′ and 5′- GAG GAG GCT GGA GAA AGT GG -3′; IRE1, 5′- ATG TGG AAG AGC CTG CCT TT - 3′ and 5′- CTA TCT GCA AAG GCC GAT GA -3′; GAPDH, 5′- ACC CAG AAG ACT GTG GAT GG -3′ and 5′- CAT ACC AGG AA ATG AGC TTG AC -3′. Twenty five PCR cycles, each consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C

and extension for 1 min at 72 °C, were carried. PCR products were analyzed by agarose gel electrophoresis.

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