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The Transcription Factor p8 Regulates Autophagy in Response to Palmitic Acid Stress via a Mammalian Target of Rapamycin (mTOR)-independent Signaling Pathway*

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Autophagy is an evolutionarily conserved degradative process that allows cells to maintain homoeostasis in numerous physiological situations. This process also functions as an essential protective response to endoplasmic reticulum (ER) stress, which promotes the removal and degradation of unfolded proteins. However, little is known regarding the mechanism by which autophagy is initiated and regulated in response to ER stress. In this study, different types of autophagy were identified in human gastric cancer MKN45 cells in response to the stress induced by nutrient starvation or lipotoxicity in which the regulation of these pathways is mammalian target of rapamycin (mTOR)-dependent or -independent, respectively. Interestingly, we found that p8, a stress-inducible transcription factor, was enhanced in MKN45 cells treated with palmitic acid to induce lipotoxicity. Furthermore, an increase in autophagy was observed in MKN45 cells stably overexpressing p8 using a lentivirus system, and autophagy induced by palmitic acid was blocked by *p8* RNAi compared with the control. Western blotting analyses showed that autophagy was regulated by p8 or mTOR in response to the protein kinase-like endoplasmic reticulum kinase/activating transcription factor 6-mediated ER stress of lipotoxicity or the parkin-mediated mitochondrial stress of nutrient starvation, respectively. Furthermore, our results indicated that autophagy induced by palmitic acid is mTOR-independent, but this autophagy pathway was regulated by p8 via p53- and PKCα-mediated signaling in MKN45 cells. Our findings provide insights into the role of p8 in regulating autophagy induced by the lipotoxic effects of excess fat accumulation in cells.

Autophagy is a phenomenon in which cytoplasmic components are delivered to the lysosomes for bulk degradation in response to prolonged starvation periods, nutritional fluctuations in the environment, developmental tissue remodeling, organelle quality control, or immune responses (1–3). Recent studies have shown that the dysregulation of autophagy is implicated in the physiopathology of major diseases such as cancer, neurodegenerative disorders, and diabetes (4–8). The autophagy process involves more than 30 autophagy-related genes and can be divided into several physiologically continuous steps (including induction, cargo recognition and packaging, and vesicle formation and breakdown) (9–11). As a key energy sensor, AMP-activated protein kinase (AMPK)[3] regulates the cellular metabolism to maintain energy homoeostasis and promotes autophagy by activating UNC-51-like kinase 1 (ULK1) through the phosphorylation of its Ser-317 and Ser-777 residues. Conversely, the central cell growth regulator mammalian target of rapamycin (mTOR) inhibits autophagy by phosphorylating Ser-757 of ULK1 and disrupting its interaction with AMPK (12–16). This coordinated pattern of phosphorylation is important for the proper control of autophagy.

Autophagy can be nonselective or selective. Non-selective bulk degradation of the cytoplasm and organelles by autophagy provides the material to support metabolism during stress. Selective autophagy involves the degradation of proteins and organelles such as the mitochondria (mitophagy), ribosomes (ribophagy), endoplasmic reticulum (ER, reticulophagy), peroxisomes (pexophagy), and lipids (lipophagy) (17–20). The ER is a complex organelle that has numerous cellular functions. The up-regulation of autophagy is an essential prosurvival response to the ER stress caused by unfolded/misfolded proteins and the aggregation of proteins, which results in protein or organelle damage (21–23). The molecular events that occur during autophagy induction include the association of autophagy-related protein 8 (ATG8)/LC3 with autophagosomal membranes through its conjugation to the lipid phosphatidylethanolamine and the formation of autophagosomes (24, 25). Cells subjected to ER stress undergo a dramatic increase in autophagy and display similar levels of lipidated ATG8/LC3 as the levels in damaged cells (21, 26–28). Additionally, light microscopy studies have revealed that ER stress induces autophagosome formation in all eukaryotes analyzed (21, 26, 29). The ER stress sensors inositol-requiring enzyme 1 (IRE1), protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) are involved in the signaling cascades inducing autophagy upon ER

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[3] The abbreviations used are: AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; ER, endoplasmic reticulum; PERK, protein kinase-like endoplasmic reticulum kinase; FFA, free fatty acid; CQ, chloroquine; PFTα, pifithrin α; EBSS, Earle's balanced salt solution; EGFP, enhanced GFP.

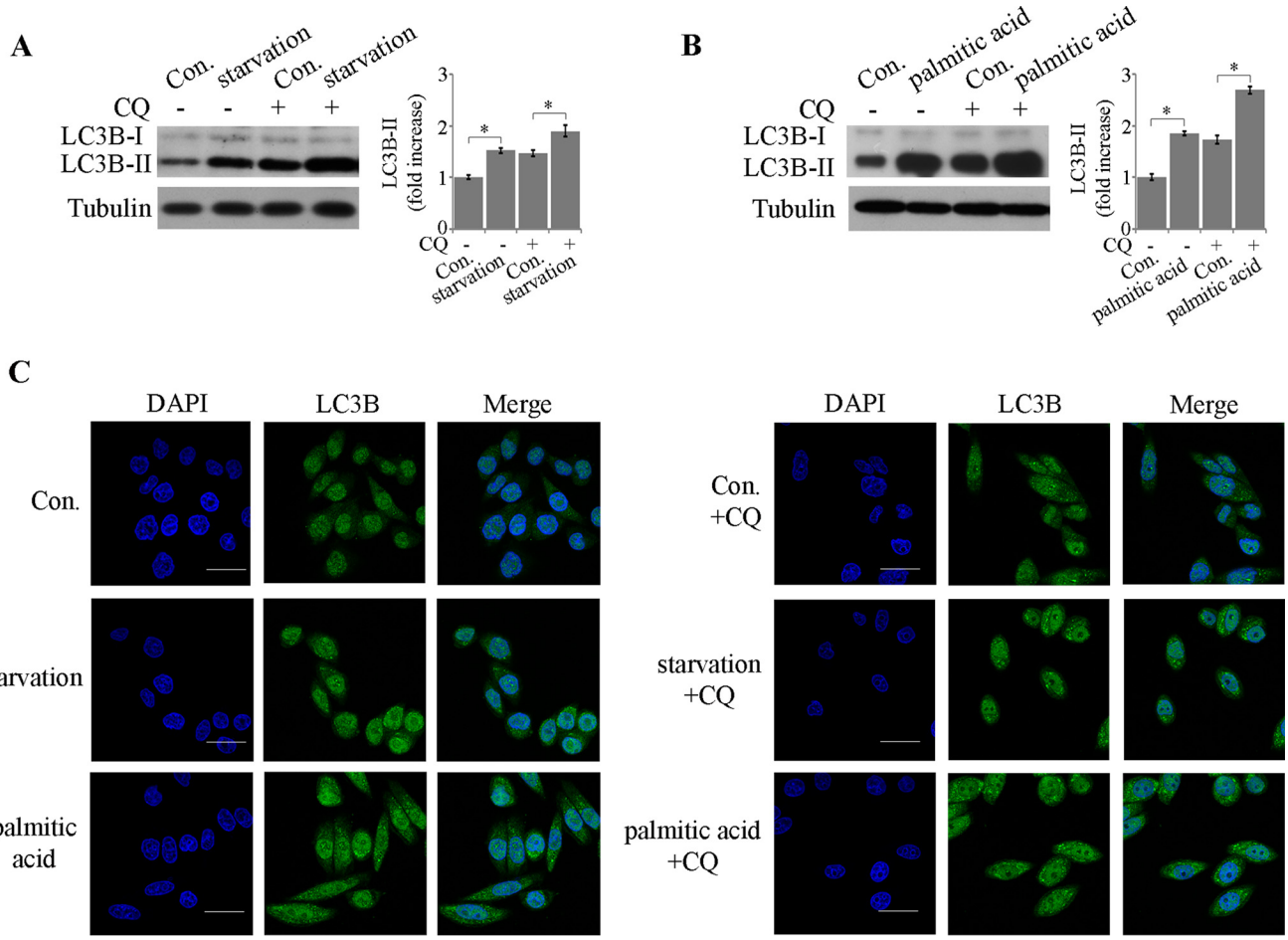

FIGURE 1. **Autophagy in response to stress induced by nutrient starvation or lipotoxicity.** To promote autophagosome generation, cells were treated with 10 μM CQ for 4 h before collection. *A*, Western blotting analysis of LC3B in control (*Con*) and nutrient-starved MKN45 cells. Nutrient starvation was induced by culturing the cells in EBSS for 6 h. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *B*, Western blotting analysis of LC3B in lipotoxic stress MKN45 cells. Lipotoxicity was induced by treating the cells with 0.25 mM palmitic acid for 6 h. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *C*, analysis of autophagy by observation of the immunofluorescence of LC3B (*green*) in MKN45 cells treated as described in *A* and *B*. The nuclei were counterstained with DAPI (*blue*). *Scale bars* = 30 μm.

stress (reticulophagy) (30–33). However, the effects of ER stress on the transcriptional regulation of the autophagy machinery or the mechanisms by which autophagy is initiated have not yet been characterized in detail.

The transcription factor p8 (or nuclear protein transcriptional regulator 1 (NUPR1)) was first identified in rats in 1997 and found to be overexpressed in acinar cells during the acute phase of pancreatitis (34). The *p8* gene is overexpressed during endothelin-induced mesangial cell hypertrophy and in the diabetic kidney (35). As a key player in the cellular stress response, p8 plays a role in several physiological and physiopathological processes and is also involved in autophagy induced by lipopolysaccharide treatment of vascular endothelial cells (36, 37). Autophagy is also controlled by p53 in a dual fashion, whereby p53 induces this process in the nuclei and suppresses it in the cytoplasm (38). A previous study has revealed the existence of a regulatory loop between the expression levels of p8 and p53 in mouse embryonic fibroblasts (39). Furthermore, p8 forms a complex with p53, regulates p21 transcription, and rescues breast epithelial cells from doxorubicin-induced genotoxic stress (40).

Lipotoxicity refers to the cytotoxic effects caused by lipid accumulation, and studies of animal models have suggested a link between ectopic lipid accumulation, cell death, and organ dysfunction (41). Elevated circulating free fatty acid (FFA) levels precede the onset of diabetes and heart failure and are associated with steatosis and the subsequent apoptosis of β cells and cardiac myocytes, respectively (42, 43). Metabolic labeling studies have demonstrated that the FFA palmitate is incorporated rapidly into phosphatidylcholine in the ER, leading to a significant increase in the saturation of the ER membrane phospholipids (44). These changes result in ER swelling and escape of the protein-folding chaperones into the cytosol, suggesting that FFAs compromise ER membrane structure and integrity (45). Supplementation of cultured cells with FFAs produces oxidative stress, and treatment of these cells with antioxidants inhibits FFA-induced caspase-3 activation, ER dysfunction, and cell death (46–48). Supplementation of cultured fibroblasts, myoblasts, and β cells with FFAs leads to alterations in the structure and function of the ER that precede the activation of ER stress responses (49). Autophagy may play an important role in the response to ER stress and has been implicated as a con-

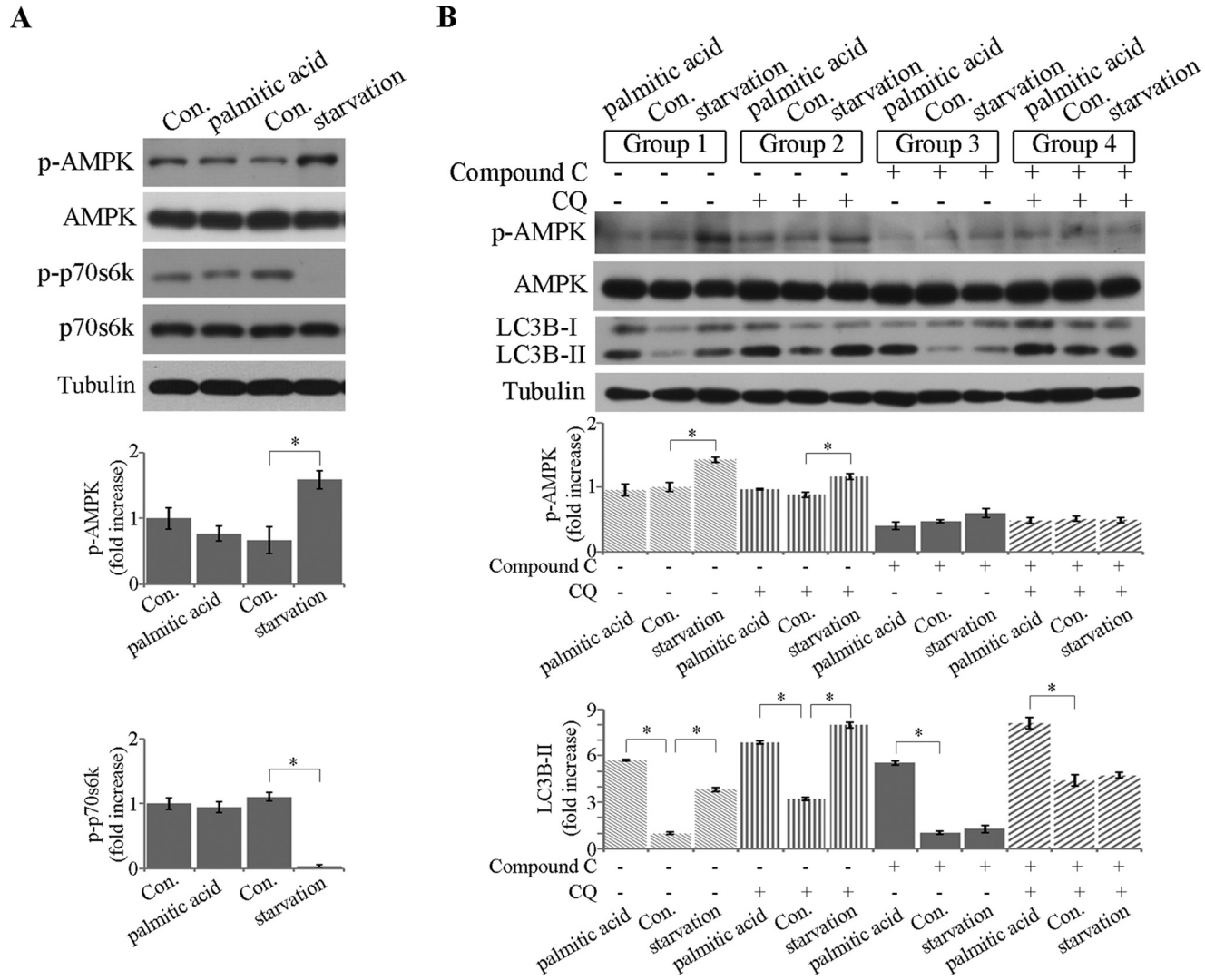

FIGURE 2. **Expression of AMPK/mTOR in response to stress induced by nutrient starvation or lipotoxicity.** *A*, Western blotting analyses of AMPK/mTOR in control (*Con*) and nutrient-starved cells and palmitic acid-treated cells. *B*, Western blotting analyses showing the effects of a p-AMPK inhibitor (compound C) on autophagy caused by pretreatment of MKN45 cells with palmitic acid or EBSS for 6 h. Compound C and/or CQ was added 4 h prior to collection. The four groups (no treatment with compound C and CQ (*group 1*, *lanes 1–3*), no treatment with compound C but treatment with CQ (*group 2*, *lanes 4–6*), treatment with compound C but no treatment with CQ (*group 3*, *lanes 7–9*), and treatment with both compound C and CQ (*group 4*, *lanes 10–12*)) were separated in this figure, and each group contained palmitic acid-treated, control, and EBSS-treated cells. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$.

tributing factor to apoptosis and cell death (27, 30). A previous report has shown that the induction of autophagy by palmitic acid is regulated via a PKC-mediated signaling pathway that is independent of mTOR (50).

In this study, the results demonstrated that autophagy induced by nutrient starvation or lipotoxicity was controlled by the mTOR or p8 signaling pathway, respectively. Notably, p8 responded to ER stress and up-regulated autophagy via the p53-PKCα-mediated signaling pathway. Overall, the results presented here indicated that the modulation of autophagy by p8 was involved in the ER stress response of cells.

Experimental Procedures

Reagents and Antibodies—Chloroquine (CQ) and palmitic acid (Sigma) and pifithrin α (PFTα) (Selleckchem, Houston, TX) were used. The primary antibodies used in this study were as follows: polyclonal anti-p-AMPK (Epitomics, Burlingame, CA, catalog no. 3930-1), polyclonal anti-p-p70s6k (Cell Signaling Technology, Leiden, The Netherlands, catalog no. 9205s), polyclonal anti-AMPK (Cell Signaling Technology, catalog no. 2532), polyclonal anti-p70s6k (Epitomics, catalog no. 1175-1), polyclonal anti-α-tubulin (Sigma, catalog no. T6199), polyclonal anti-p8 (Abcam, Cambridge, UK, catalog no. ab46889; Santa Cruz Biotechnology, catalog no. sc-30184), polyclonal anti-LC3B (Sigma, catalog no. L7543), polyclonal anti-p-PERK (Cell Signaling Technology, catalog no. 3179), polyclonal anti-PERK (Cell Signaling Technology, catalog no. 5683), polyclonal anti-ATF6 (Abcam, catalog no. ab37149), polyclonal anti-parkin (Abcam, catalog no. ab77924), polyclonal anti-p-PKCα (Millipore, Nottingham, UK, catalog no. 06-822), polyclonal anti-PKCα (Cell Signaling Technology, catalog no. 2056), polyclonal anti-p-p53 (Cell Signaling Technology, catalog no. 9284P), and polyclonal anti-p53 (Epitomics, catalog no. 1026-1).

Cell Lines and Cell Culture—Human gastric cancer cells (MKN45) and HeLa cells were maintained separately in RPMI 1640 medium (Corning, New York, NY) or DMEM (Corning)

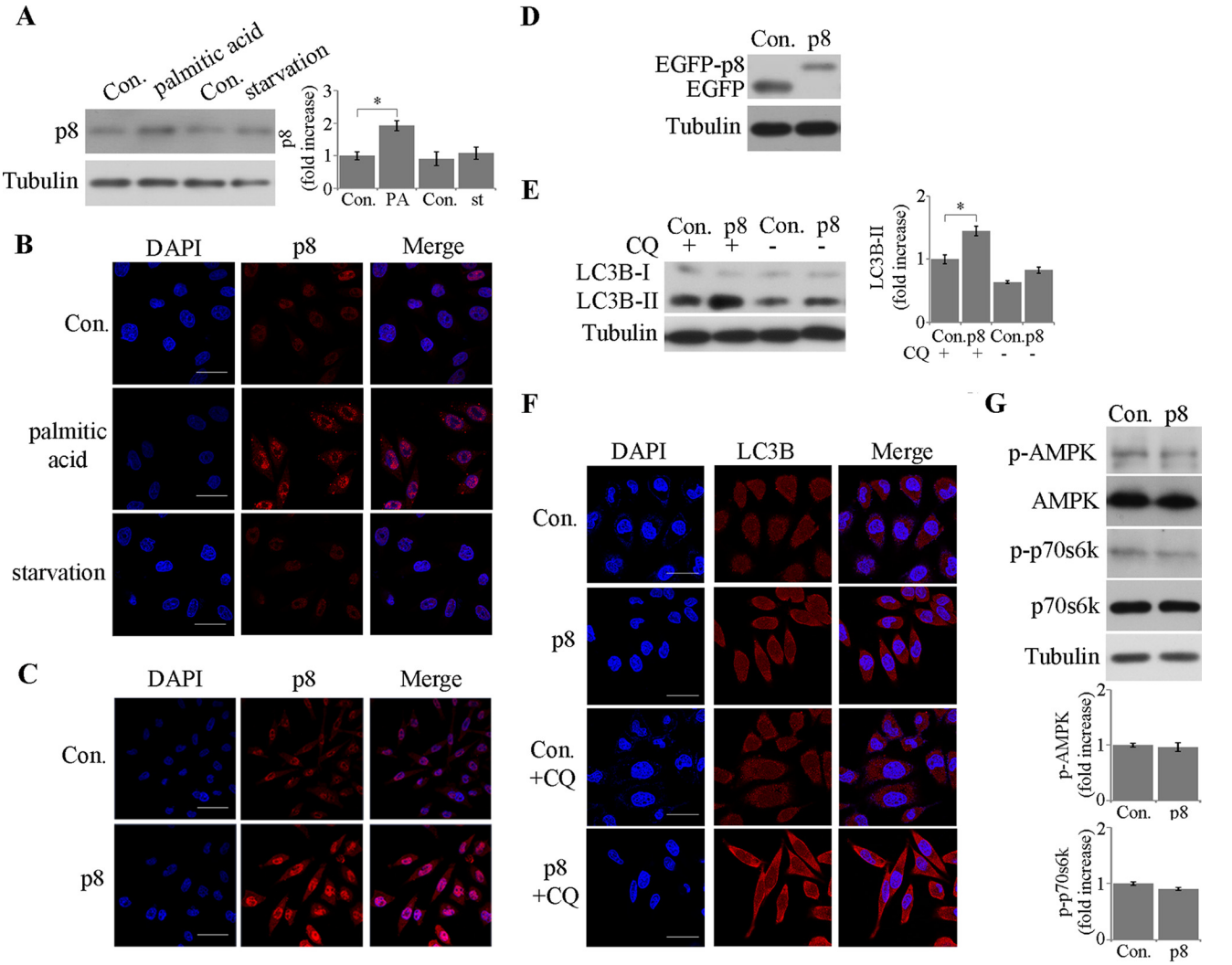

FIGURE 3. **Expression of the transcription factor p8 in response to stress induced by nutrient starvation or lipotoxicity.** *A*, representative Western blotting analyses and quantitative data of p8 in control (*Con*), palmitic acid (*PA*)-treated, and nutrient-starved (*st*) MKN45 cells. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *B*, immunofluorescence analyses of p8 expression (*red*) in MKN45 cells treated with palmitic acid for 6 h or with EBSS for 6 h. The nuclei were counterstained with DAPI (*blue*). *Scale bars* = 30 μm. *C*, immunofluorescence of p8 in the MKN45 cells stably expressing EGFP-tagged p8 or EGFP as a control. The nuclei were counterstained with DAPI (*blue*). p8 is shown in *red*. *Scale bars* = 50 μm. *D*, Western blotting analysis of EGFP in MKN45 cells stably expressing EGFP-tagged p8 or EGFP (*Con*) alone. *E*, Western blotting analysis of LC3B in MKN45 cells stably expressing EGFP-tagged p8 or EGFP (*Con*) alone. To promote autophagosome generation, the cells were treated with 10 μM CQ for 4 h before collection. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *F*, immunofluorescence of LC3B (*red*) in MKN45 cells stably expressing EGFP-tagged p8 or EGFP (*Con*) alone. The nuclei were counterstained with DAPI (*blue*). CQ was added 4 h before collection. *Scale bars* = 30 μm. *G*, Western blotting analyses of AMPK (*p-AMPK*) and p70s6k (*p-p70s6k*) in MKN45 cells overexpressing p8. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$.

containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Sigma), which was defined as complete medium in this study. The cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Transfection—p53 siRNA (Santa Cruz Biotechnology, catalog no. sc-29435, a pool of 3 target-specific 19- to 25-nt siRNAs) and *p8* siRNAs (Ribobio, catalog no. Q000004927-1-A, a kit containing three target-specific, 19- to 25-nt siRNAs) were used to knock down *p53* and *p8* gene expression. The *p8*, *p53*, and control siRNA (100 pM) were transfected into MKN45 cells using siRNA transfection reagent (Santa Cruz Biotechnology) 42 h before palmitic acid treatment, according to the instructions of the manufacturer. The cells were collected after palmitic acid treatment for 6 h.

Lentiviral Package and Transduction—On the basis of the sequence of the human *p8* gene in GenBank, the EGFP-p8 overexpression plasmid was synthesized and transfected with a viral packaging plasmid (pVSVG and Δ8.91) into HEK293T cells overnight, and the viral supernatant was collected 48 h later. The viral supernatant was filtered through a 0.45-μm filter, and the MKN45 cells were infected in the presence of 5 μg/ml Polybrene (Sigma) and selected for 3–7 days, depending on the level of EGFP expression. Fluorescent cell sorting was used to isolate EGFP-p8-positive cells.

Western Blotting—Total cell lysates were prepared from MKN45 and HeLa cells and quantified using the Bradford method. Each total protein sample (4 μg) was separated on SDS-PAGE gels and then transferred to PVDF membranes (Bio-Rad). After incubation of the membranes with primary antibodies at 4 °C overnight, the specific proteins were detected using the BM chemiluminescence Western blotting kit (Roche).

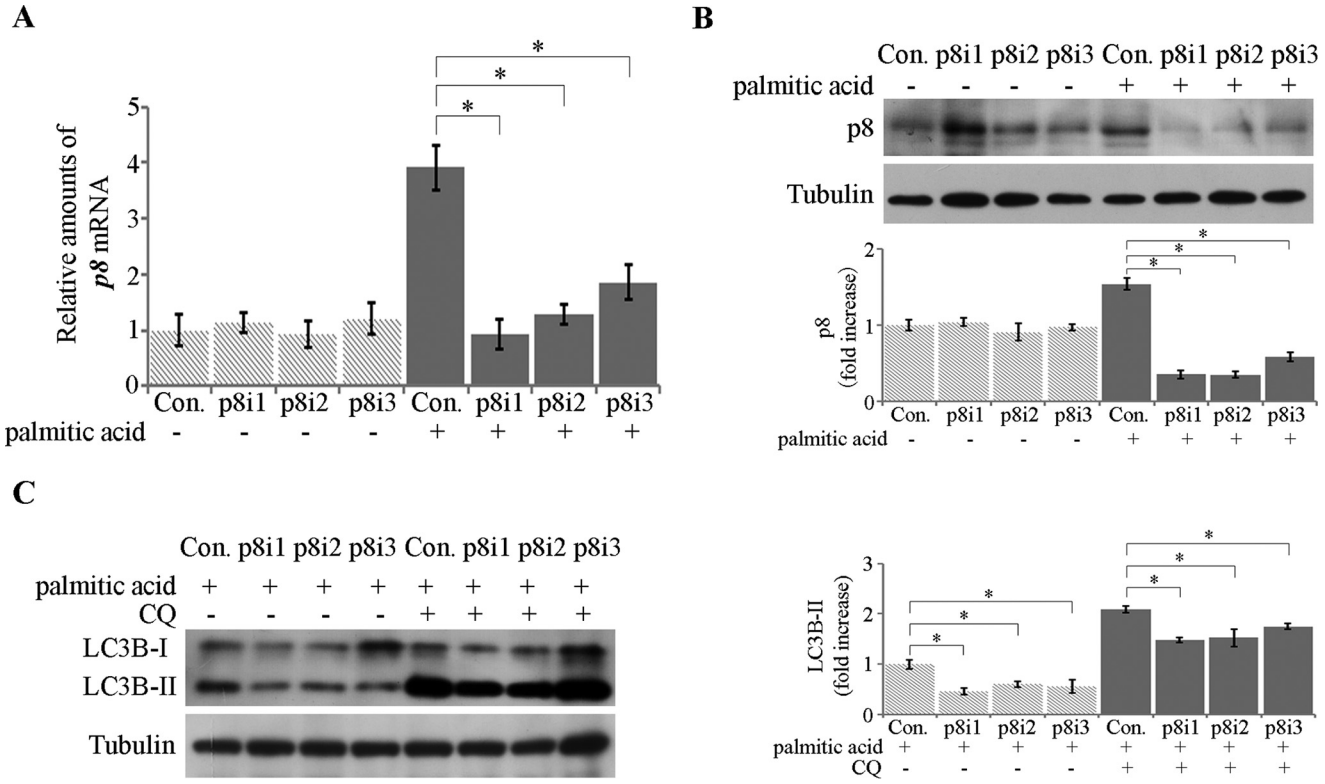

FIGURE 4. **Autophagy was down-regulated by knockdown of *p8* in cells pretreated with palmitic acid.** *A*, *p8*-specific siRNAs (100 pM) were transfected into MKN45 cells with siRNA transfection reagent for 48 h. Quantitative RT-PCR of *p8* in control (*Con*) and *p8* interference (*p8i1*, *p8i2*, and *p8i3*) MKN45 cells with or without 0.25 mM palmitic acid treatment for 6 h was performed. *B*, representative Western blotting analyses and quantitative data of p8 in control and p8 interference MKN45 cells with or without 0.25 mM palmitic acid treatment for 6 h. *C*, representative Western blotting analyses and quantitative data of LC3B in control and p8 interference MKN45 cells after 0.25 mM palmitic acid treatment for 6 h. To promote autophagosome generation, the cells were treated with 10 μM CQ for 4 h before collection. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$.

Immunofluorescence—MKN45 cells were fixed with 4% paraformaldehyde at 4 °C overnight and then washed with PBS. Fixed cells were blocked in antibody dilution buffer (PBS containing 0.25% Triton X-100 and 1% bovine serum albumin), and all subsequent staining was performed in the same buffer. The cells were labeled with primary antibody for 2 h and subsequently with Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (Invitrogen Life Technologies) or Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (Invitrogen Life Technologies) for 1.5 h. The nucleus was stained by incubation with DAPI for 15 min at room temperature.

Results

Autophagy Induced by Nutrient Starvation or Lipotoxicity Is AMPK/mTOR-dependent or -independent, Respectively—Recent studies have shown that the cytotoxic effects of fatty acids and nutrient starvation can induce autophagy (51–54). However, it is not known whether the two different stresses induce autophagy in the same manner. To answer this question, nutrient starvation and lipotoxicity were used to induce autophagy in MKN45 cells. Nutrient starvation was induced by culturing the cells in Earle's balanced salt solution (EBSS), which lacks free amino acids and glucose, and lipotoxicity was induced by supplementation of the culture medium with palmitic acid. To measure the autophagic flux in the cells, we used CQ to block the lysosomal function and the late degradation stage of autophagy (55). The results of Western blotting and immunofluorescence showed that cells cultured in the EBSS had a higher amount of lipidated LC3B than control cells cultured in complete medium, indicating that autophagy was increased in response to nutrient starvation for 6 h (Fig. 1, *A* and *C*). Similarly, autophagy was also induced in cells treated with palmitic acid for 6 h (Fig. 1, *B* and *C*).

The AMPK/mTOR signaling pathway has been well established as the key regulator of the autophagic process. To further examine the differences in autophagy induced by nutrient starvation and lipotoxic stress, the levels of AMPK/mTOR were analyzed by Western blotting. Furthermore, p70s6k is a direct substrate of mTOR, and its phosphorylation status can be used as an indicator of mTOR activity (56). The results showed that the level of p-AMPK was increased in response to nutrient starvation and that the level of p-p70s6k was decreased, whereas palmitic acid-induced lipotoxic stress in MKN45 cells had no effect on the levels of p-AMPK or p-p70s6k (Fig. 2*A*). Furthermore, MKN45 cells were treated with compound C, an inhibitor of p-AMPK. As shown in Fig. 2*B*, autophagy was increased in both palmitic acid- and EBSS-treated cells without compound C treatment, and the level of p-AMPK was increased under nutrition starvation but not under lipotoxicity stress (Fig. 2*B*, *groups 1* and *2*, *lanes 1–6*). Moreover, autophagy was increased in lipotoxic cells but not in starved cells under treatment with compound C (Fig. 2*B*, *groups 3* and *4*, *lanes 7–12*). The results indicated that autophagy induced by

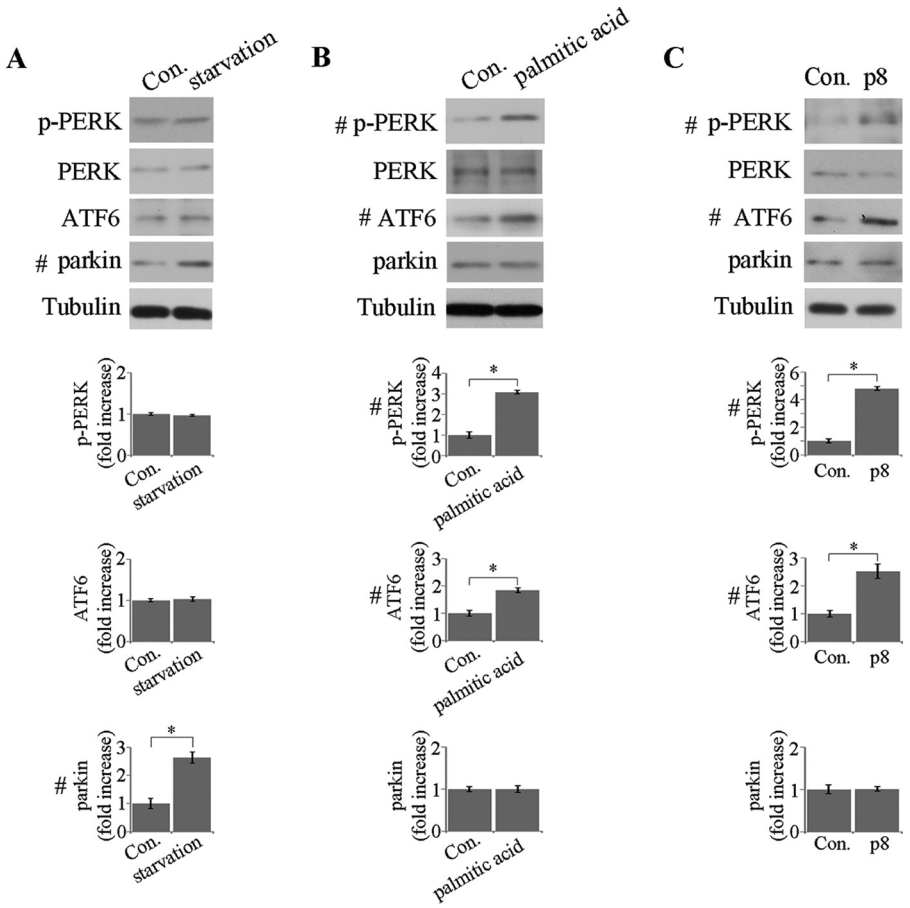

FIGURE 5. **The characteristics of autophagy induced by palmitic acid or nutrient starvation.** *A–C*, Western blotting analyses of phosphorylated PERK and ATF6 (both of which are involved in ER stress-mediated autophagy) and parkin (which is involved in mitochondrial stress-mediated autophagy) in MKN45 cells cultured in EBSS (starvation) for 6 h (*A*), in MKN45 cells treated with 0.25 mM palmitic acid for 6 h (*B*), and in MKN45 cells stably overexpressing p8 (*C*). Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. #, proteins with significant changes. *Con*, control.

nutrient starvation was regulated by the AMPK/mTOR signaling pathway and autophagy induced by lipotoxicity was AMPK/mTOR-independent.

Autophagy Induced by Lipotoxicity Is Controlled by p8 and Is Independent of the AMPK/mTOR Signaling Pathway—As a key player in the cellular stress response, p8 is also involved in autophagy induced by lipopolysaccharide treatment of vascular endothelial cells (36–37). To further understand the autophagy mechanism induced by nutrient starvation and lipotoxic stress, p8 was analyzed in the two types of autophagy by Western blotting and immunofluorescence. As shown in Fig. 3, *A* and *B*, palmitic acid treatment increased the p8 protein level. In contrast, starvation-induced autophagy had no effect on the p8 level in MKN45 cells. This result suggested that p8 was involved in induction of autophagy by lipotoxicity but not nutrient starvation.

To explore the function of p8 in the regulation of autophagy, we constructed a MKN45 cell line stably overexpressing p8 using a lentivirus system in which EGFP-fused p8 was overexpressed successfully (Fig. 3, *C* and *D*), and the level of LC3B was increased in the p8-overexpressing MKN45 cells (Fig. 3*E*). This result was confirmed by immunofluorescence, which showed an increased level of autophagy in cells overexpressing p8 (Fig. 3*F*). On the basis of these results, we concluded that p8 regulated autophagy in response to lipotoxic stress in MKN45 cells. Furthermore, we examined whether p8-regulated autophagy was dependent on the AMPK/mTOR pathway. As shown in Fig. 3*G*, p8 overexpression did not affect the levels of p-AMPK or p-p70s6k in MKN45 cells.

RNAi was used to knock down *p8* gene expression to elucidate the function of p8 in the regulation of autophagy induced by lipotoxicity. The mRNA and protein levels of p8 were not decreased after *p8* RNAi without treatment of palmitic acid because of the low basal level of *p8* in MKN45 cells (Fig. 4, *A* and *B*). However, the mRNA level of *p8* was reduced 75%, 66%, and 53%, respectively, and the protein level of p8 was also reduced 78%, 78%, and 62%, respectively, in palmitic acid-treated cells (Fig. 4, *A* and *B*). Furthermore, Western blotting analysis showed that the protein level of LC3B in palmitic acid-treated cells was reduced after *p8* RNAi compared with control cells (Fig. 4*C*). Therefore, autophagy was down-regulated by knockdown of *p8* in cells pretreated with palmitic acid. On the other hand, p8 expressed a very low basal level in MKN45 cells without treatment of palmitic acid. The results indicated that p8 regulated lipotoxicity-induced autophagy but was not involved in the basal regulation of autophagy.

Autophagy Regulated by p8 or AMPK/mTOR Is in Response to the PERK/ATF6-mediated ER Stress of Lipotoxicity or the Parkin-mediated Mitochondrial Stress of Nutrient Starvation, Respectively—To further examine the differences between the two types of autophagy induced by nutrient starvation or lipotoxic stress, the levels of phosphorylated PERK (p-PERK) and

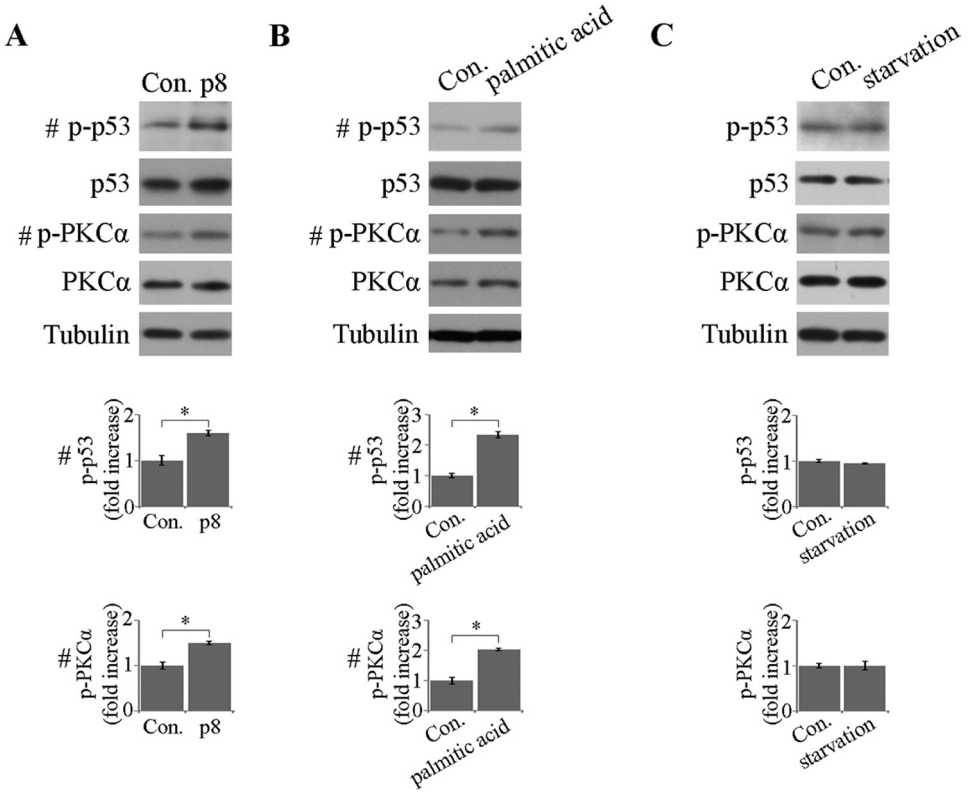

FIGURE 6. **Palmitic acid stress-induced autophagy is regulated by p8 via effects on the p53 and PKCα signaling pathways.** *A–C*, Western blotting analyses of p53 (*p-p53*) and PKCα (*p-PKCα*) in MKN45 cells stably overexpressing p8 (*A*), in MKN45 cells treated with 0.25 mM palmitic acid for 6 h (*B*), and in MKN45 cells cultured in EBSS for 6 h (*C*). Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. #, proteins with significant changes. *Con*, control.

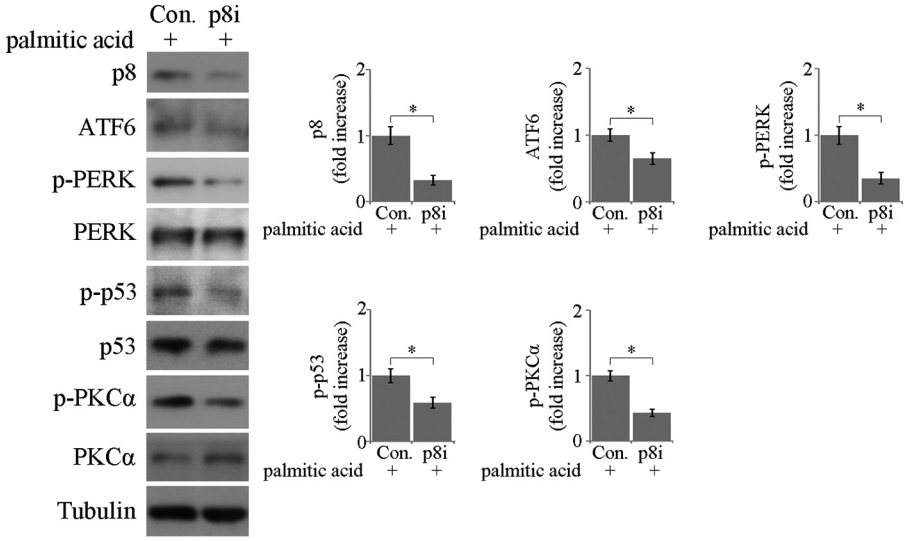

FIGURE 7. **p8 regulates autophagy induced by palmitic acid via the p53-PKCα signaling pathways.** Shown are representative Western blotting analyses and quantitative data of p8, ATF6, PERK (*p-PERK*), p53 (*p-p53*) and PKCα (*p-PKCα*) in control (*Con*) and *p8* interference (*p8i*) MKN45 cells after 0.25 mM palmitic acid treatment for 6 h. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$.

ATF6 (both of which were involved in ER stress-induced autophagy (30–33), and parkin (which was involved in mitochondrial stress-induced autophagy (57–60)) were determined in MKN45 cells exposed to both stresses. Western blotting analyses revealed that nutrient starvation increased the level of parkin but did not affect the levels of p-PERK or ATF6 (Fig. 5*A*). By contrast, palmitic acid treatment or overexpression of p8 increased the levels of p-PERK and ATF6 but had no effect on the level of parkin (Fig. 5, *B* and *C*). These results suggested that autophagy induced by palmitic acid was an ER-stress response regulated by the p8 signaling pathway, whereas autophagy induced by nutrient starvation was a mitochondrial-stress response controlled by the AMPK/mTOR signaling pathway.

Palmitic Acid Stress-induced Autophagy Is Regulated by p8 via p53 and PKCα Signaling—In contrast to the pathway involved in the regulation of nutrient starvation-induced

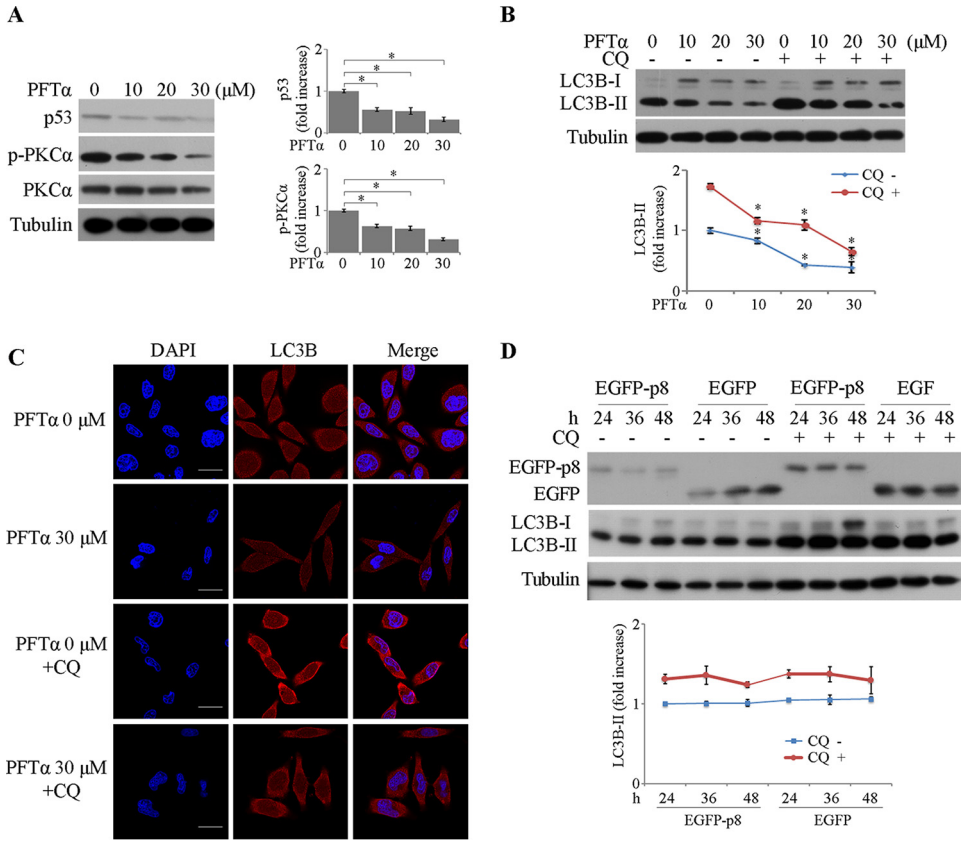

FIGURE 8. **p53 is involved in the p8-regulated autophagy signaling pathway.** To promote autophagosome generation, cells were treated with 10 μM CQ for 4 h before collection. *A*, Western blotting analyses of p53 and PKCα (*p-PKCα*) in MKN45 cells overexpressing p8 treated with the p53 inhibitor PFTα (0–30 μM) for 24 h. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *B*, Western blotting analysis of LC3B in MKN45 cells overexpressing p8 treated with PFTα (0–30 μM) for 24 h. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *C*, immunofluorescence analyses of autophagy (LC3B expression, *red*) in MKN45 cells overexpressing p8 treated with PFTα (30 μM) for 24 h or left untreated. The nuclei were counterstained with DAPI (*blue*). *Scale bars* = 30 μm. *D*, HeLa cells were transfected with EGFP-fused p8 for 24–48 h, and Western blotting analysis of LC3B was performed. Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$.

autophagy, the signaling pathway involved in the regulation of ER stress-induced autophagy remains largely unclear. Overexpression of p8 or exposure of MKN45 cells to palmitic acid, but not to nutrient starvation, increased the phosphorylation levels of both p53 (p-p53) and PKCα (p-PKCα) (Fig. 6, *A*–*C*).

To elucidate the p8-p53-PKCα signaling pathway, a *p8* siRNAs was used to knock down *p8* gene expression in which the mRNA level of *p8* was reduced 75% and the protein level of p8 was also reduced 78% compared with control cells (Fig. 4, *A* and *B*). The results indicated that the levels of ATF6, p-PERK, p-p53, and p-PKCα were decreased significantly after *p8* RNAi with pretreatment of palmitic acid in MKN45 cells (Fig. 7).

To confirm that autophagy regulated by p8 (Fig. 3, *E* and *F*) is mediated by p53 and PKCα, p53 was inhibited by PFTα (a p53 inhibitor), and the level of p-PKCα was decreased in p8-overexpressing MKN45 cells (Fig. 8*A*). The results showed that the autophagy induced by p8 overexpression was decreased, using PFTα by Western blotting and immunofluorescence analyses (Fig. 8, *B* and *C*). Furthermore, the same results were observed in other cell lines, including HT1080, MCF7, and HEK293T (data not shown), but with the exception of the HeLa cell line. The HeLa cell line was therefore used as a model to study the autophagy regulation pathway mediating by p53 because of the functional deletion of p53. The results showed that p8 overexpression in p53-dysfunctional HeLa cells had no effect on the level of LC3B (Fig. 8*D*), in contrast to its effects on MKN45 cells (Fig. 3, *E* and *F*).

To further demonstrate that the autophagy mechanism induced by palmitic acid was mediated by p53, we transfected *p53* siRNA, which is a pool of three *p53* target-specific, 19- to 25-nt siRNAs, into palmitic acid-treated and control MKN45 cells and found that both the mRNA and protein levels of p53 were decreased significantly (Fig. 9, *A* and *B*). Meanwhile, Western blotting analyses showed that autophagy was reduced and that the levels of p53, ATF6, p-PERK, and p-PKCα were decreased after *p53* RNAi in palmitic acid-treated MKN45 cells (Fig. 9, *C* and *D*). Overall, these results indicated that p8 regulated autophagy in response to ER stress via the p53 and PKCα signaling pathways.

On the basis of the results described above, we propose a tentative model of palmitic acid stress-mediated autophagy and regulation of the p8 signaling pathway (Fig. 10). According to this model, p8 expression is up-regulated in response to ER stress such as lipotoxicity and activates p53 via phosphorylation. In turn, p-p53 induces the phosphorylation and activation of PKCα, leading to increased expression of p-PERK and ATF6 and subsequent initiation of autophagy.

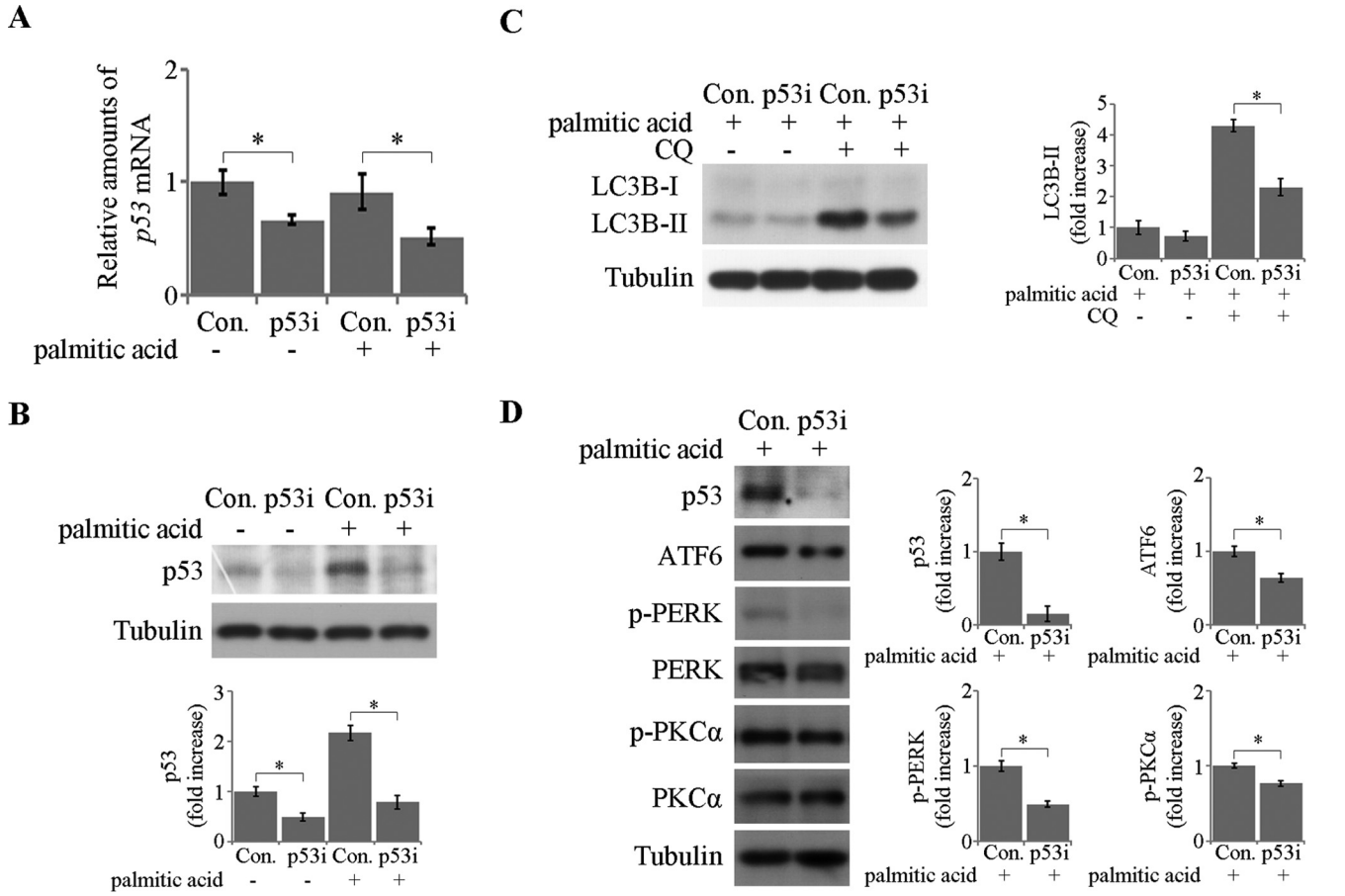

FIGURE 9. **Autophagy was down-regulated by knockdown of *p53* in cells pretreated with palmitic acid.** Quantitative data of optical band densitometry are shown. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$. *A*, *p53*-specific siRNA (100 pM) was transfected into MKN45 cells with siRNA transfection reagent (Santa Cruz Biotechnology) for 48 h. Quantitative RT-PCR of *p53* in control (*Con*) and *p53* interference (*p53i*) MKN45 cells with or without treatment of 0.25 mM palmitic acid for 6 h was performed. *B*, representative Western blotting analyses and quantitative data of p53 in control and *p53* interference MKN45 cells with or without 0.25 mM palmitic acid treatment for 6 h. *C*, representative Western blotting and quantitative data of LC3B in control and *p53* interference MKN45 cells after 0.25 mM palmitic acid treatment for 6 h. To promote autophagosome generation, the cells were treated with 10 μM CQ for 4 h before collection. *D*, representative Western blotting and quantitative data of p53, ATF6, PERK (*p-PERK*) and PKCα (*p-PKCα*) in control and *p53* interference MKN45 cells after 0.25 mM palmitic acid treatment for 6 h.

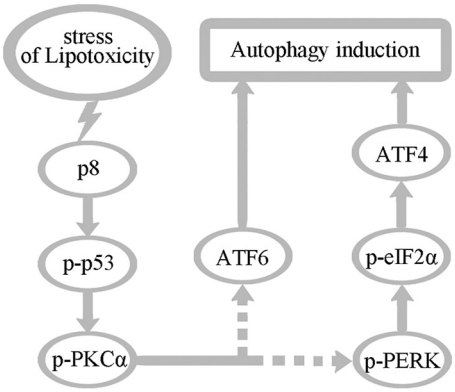

FIGURE 10. **Proposed model of the p8-regulated autophagy signaling pathway in response to palmitic acid stress.**

Discussion

Accumulating studies have shown that the regulation of autophagy is implicated in the physiopathology of major diseases such as cancer and neurodegenerative disorders. Specifically, autophagy induced by lipotoxicity has been implicated as one of the contributing factors to diseases such as obesity, diabetes, and non-alcoholic fatty liver, which refers to the cytotoxic effects of excess fat accumulation in cells. In this study, we identified an mTOR-independent signaling pathway in which p8 regulated autophagy in response to ER stress induced by lipotoxicity. Moreover, we showed that palmitic acid induced autophagy by p53-PKCα-mediated signaling, which differed from mTOR-dependent autophagy in response to stress induced by nutrient starvation in MKN45 cells. Furthermore, similar results were observed in other cell lines, including a fibrosarcoma cell line (HT1080), breast cancer cell line (MCF7), and embryonic kidney cell line (HEK293T) (data not shown). It is believed that autophagy in response to different types of cellular stress is regulated by various molecular signaling pathways.

Autophagy is a selective process that degrades various organelles in response to specific stimuli. Maintenance of organelle quality and quantity is critical for cellular homeostasis and adaptation to variable environments. Okamoto (20) has demonstrated that this type of control is achieved by selective elimination of organelles, such as the peroxisome, mitochondrion, lipid droplet (the structure surrounded by a phospholipid monolayer), lysosome, nucleus, and ER, and even nonmembranous structures, such as ribosomes, via autophagy, termed organellophagy . Sasaki and Yoshida (61) have summarized the

homeostatic mechanism that regulates the capacity of each organelle according to the cellular demands as organelle autoregulation . Both nutrient starvation and lipotoxicity have been shown to cause an imbalance in cellular homeostasis. The difference is that nutrient starvation mainly causes mitochondrial damage, where ATP is synthesized through oxidative phosphorylation, whereas the lipotoxic effect causes ER damage, where the secretory and membrane proteins are synthesized and folded with the assistance of the ER chaperones. The lipotoxicity caused by overloading of fibroblasts, myoblasts, and β cells with saturated FFAs leads to alterations in the structure and function of the ER that precede activation of the ER stress response (45, 62). Autophagy induced by ER stress may play an important role in maintaining ER homoeostasis by segregating and/or degrading a part of this structure in response to various ER stresses (30).

Previous studies have shown that autophagy was positively regulated by p8 in human glioma cells and umbilical vein endothelial cells; however, p8 deficiency resulted in increased autophagy in H9C2 and U20S cells (37, 63, 64). These findings may suggest a cell-specific role of p8 in autophagy. Here we found that p8 overexpression up-regulated autophagy in MKN45 cells. However, p8 was not involved in the induction of autophagy by nutrient starvation, which activated the AMPK/mTOR signaling pathway. Therefore, we concluded that p8 controlled autophagy to promote cell survival in response to specific environmental stresses such as lipotoxicity. This proposal may also be supported by a previous study in which *p8* mRNA expression in pancreatic cells was induced in response to the lipotoxic stress of lipopolysaccharides (65). Overall, our results indicated that there were two different regulation pathways of autophagy in response to palmitic acid treatment and nutrient starvation: the p8- and AMPK/mTOR-mediated pathways.

In mammalian cells, ATF6 and PERK sensed ER stress and triggered a general transcriptional response that affected the level of ATG12, which was involved in autophagy. The ATG12-ATG5 (ATG16) complex facilitated the lipidation of ATG8 and subsequent autophagy induction (30). However, it is not clear how ATF6 and PERK are activated in response to ER stress and whether the activation signaling pathway is dependent on the stimulation of AMPK and the inhibition of mTOR kinases. Here we found that p8 regulated the activation of ATF6 and PERK via the p53 and PKCα signaling pathways in response to palmitic acid-mediated stress. Moreover, this process was independent of mTOR signaling. These findings provide the basis for further studies of mTOR-independent autophagy, such as the signaling pathway induced in response to lipotoxicity.

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