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Oxysterol-Binding Protein-Related Protein 8 Inhibits Gastric Cancer Growth Through Induction of ER Stress, Inhibition of Wnt Signaling, and Activation of Apoptosis

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Gastric cancer (GC) is the third leading cause of cancer-related mortality worldwide. Oxysterol-binding protein-related protein 8 (ORP8) functions as a sterol sensor that regulates a number of cellular functions. We showed that ORP8 expression was significantly lower in GC tissues and cells. Overexpression of ORP8 significantly inhibited GC cell proliferation in several GC cells. The formation of colonies in AGS cells was inhibited by the overexpression of ORP8. Moreover, overexpression of ORP8 significantly decreased implanted tumor growth in nude mice. Overexpression of ORP8 resulted in a significant increase in CHOP and GRP78 expression and the phosphorylation of PERK, indicating the occurrence of ER stress. Inhibition of ER stress by 4-PBA notably suppressed overexpression of ORP8-induced decrease of GC cell proliferation, formation of colonies, and implanted tumor growth. Overexpression of ORP8 resulted in a significant decrease in Wnt3a and β-catenin expression, and activation of Wnt signaling by HLY78 markedly blocked overexpression of ORP8-induced decrease in GC cell proliferation, formation of colonies, and implanted tumor growth. 4-PBA inhibited overexpression of ORP8-induced decrease in Wnt signaling. Furthermore, overexpression of ORP8 resulted in significant activation of mitochondrial apoptotic events and increase in apoptosis, which was inhibited by 4-PBA and HLY78. Induction of ER stress, inhibition of Wnt signaling, and apoptotic cell death were involved in ORP8-induced inhibition of GC cell proliferation. These findings indicate that downregulation of ORP8 plays a pivotal role in the progression of GC, and it may be a novel therapeutic target in the treatment of GC.

Key words: Oxysterol-binding protein-related protein 8 (ORP8); Gastric cancer (GC); Endoplasmic reticulum (ER) stress; Wnt signaling; Mitochondrial apoptosis

INTRODUCTION

Gastric cancer (GC) is the most common gastrointestinal tumor, representing the third leading cause of cancer-related mortality worldwide[1–3]. It is reported that GC caused 723,100 deaths worldwide in 2012[1–3]. More than 70% of new cases and deaths occur in developing countries, particularly in Eastern Asia, Eastern Europe, and South America[2]. Owing to the rapid advance in surgical techniques, therapy for nonmetastatic gastric adenocarcinoma has greatly improved. However, GC is usually diagnosed in the local advanced or metastatic stage, with a grim 5-year survival rate of less than 30%[4–6]. Adjuvant chemotherapy is commonly used in combination with surgery for the treatment of metastatic GC. The prognosis of GC patients is still poor because of the high rate of metastasis and postsurgical recurrence. However, the efficacy of chemotherapy is limited by more and more drug resistance. In recent years, although a large number of studies have been conducted to find new specific oncogenic/anti-oncogenic signaling pathways in the development of GC, few significant results have been obtained[7,8]. Therefore, it is urgent that molecular mechanisms underlying the development and progression of GC and identification of new therapeutic targets are explored.

Oxysterol-binding protein was first isolated in the 1980s and regarded as a cytoplasmic high-affinity receptor for several oxysterols[9–11]. These proteins play a role in the transport of ceramide from the endoplasmic reticulum (ER) to the Golgi apparatus for the synthesis of sphingomyelin[12]. They also act as a sterol-dependent scaffold that regulates the activity of extracellular signal-regulated kinases[13]. The mammalian oxysterol-binding protein-related protein (ORP) functions as a sterol sensor that regulates a number of cellular functions, including

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sterol and neutral lipid metabolism, vesicle transport, and cell signaling[14]. Mammalian ORP comprises a 12-member gene family[15]. ORP8 is a member of the ORP family that plays an important role in various biological processes. Yan et al. found that ORP8 decreased cholesterol efflux in macrophages by suppressing ABCA1 expression, suggesting a role for ORP8 in the development of atherosclerotic lesions[16]. Zhou et al. discovered that ORP8 downregulated SREBP-1 and SREBP-2 target genes, acting as a negative regulator of intracellular cholesterol[17]. Studies have shown that ORP8 could inhibit cell migration through interaction with nucleoporin Nup62[18], regulate the cell cycle through interaction with astrin/SPAG5[19], and transport phosphatidylserine at the ER–plasma membrane contact sites[20]. ORP8 increases the sensitivity of hepatocellular carcinoma cells to Fas-mediated apoptosis[21]. Further evidence supports that it mediates the cytotoxicity of 25-hydroxycholesterol[22]. This evidence indicates a role for ORP8 in the regulation of cell proliferation and death.

In this study, we explored the role of ORP8 in GC cell proliferation and showed that ORP8 was significantly lower in GC tissues and cells. Overexpression of ORP8 significantly inhibited GC cell proliferation and implanted tumor growth in vivo. Induction of ER stress, inhibition of Wnt signaling, and apoptotic cell death were involved in ORP8-induced inhibition of GC cell proliferation. These findings indicate that downregulation of ORP8 plays a pivotal role in the progression of GC, and it may be a novel therapeutic target in the treatment of GC.

MATERIALS AND METHODS

Chemicals and Materials

β-Actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). CHOP, GRP78, P-PERK, PERK, Wnt3a, β-catenin, cleaved caspase 3, and cleaved caspase 9 STAT3 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). MitoSOX was obtained from Invitrogen (Carlsbad, CA, USA).

Patients and Ethics Statement

Twenty-nine patients who underwent resection of primary GC at The First Affiliated Hospital of Xinxiang Medical University between October 2014 and February 2016 were included in this study. Tumor tissues and adjacent nontumor tissues were collected. Fresh tissue was obtained and stored at −80°C before use. The diagnosis of GC was established using the World Health Organization (WHO) morphological criteria. All the patients had provided informed consent, and the study was approved by The First Affiliated Hospital of Xinxiang Medical University and complied with the Declaration of Helsinki.

Cell Culture and Transfection

Human gastric epithelial cell line GES-1 and GC cells (AGS, HGC-27, MKN-1, MKN-28, MKN-45, MGC-803, and SGC-7901) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C. Lentivirus carrying ORP8 was synthesized commercially and transfected into AGS, HGC-27, and MGC-803 cells to establish GC cell lines stably expressing ORP8 according to the manufacturer's protocols.

Cell Proliferation

Cells were plated into 96-well plates and cultured in the presence or absence of 10 μM 4-PBA or HLY78 for 48 h. Cell proliferation was assayed by CCK-8 (Bioworld Technology, Inc., St. Louis Park, MN, USA). Cells were plated into 96-well plates, and cell numbers were evaluated by CCK-8 following the manufacturer's protocol. Cell proliferation was shown as folds of cell numbers versus control.

Colony Formation

Cells were plated into six-well plates and cultured in the presence or absence of 10 μM 4-PBA or HLY78 for 48 h. Cellular colony formation was evaluated. Briefly, cells were suspended in 0.5% low-melting-point agarose (Invitrogen) in 1 ml of culture medium. The cells were then placed in 2 ml of complete medium and into six-well culture plates with 0.75% agarose (Sigma-Aldrich, St. Louis, MO, USA) on the bottom layer. Cells were cultured for 2 weeks, and the colonies were stained with 0.005% crystal violet (Sigma-Aldrich) and observed using a microscope (Olympus, Tokyo, Japan).

Apoptosis

Cells were plated into six-well plates and cultured in the presence or absence of 10 μM 4-PBA or HLY78 for 48 h. Apoptosis was assessed by an In Situ Cell Death Detection Kit, Fluorescein (Roche) following the manufacturer's protocol. Detection and analysis were performed using flow cytometry (BD, C6).

Mitochondrial ROS Level

Cells were plated into special plates used for confocal microscopy and cultured in the presence or absence of 10 μM 4-PBA or HLY78 for 48 h. After treatment, cells were incubated with 1 μM MitoSOX in serum-free medium for 20 min at 37°C in a humidified incubator. Fluorescence was recorded using a confocal microscope (Olympus).

Mitochondrial Oxygen Consumption

Cells were plated into six-well plates and cultured in the presence or absence of 10 μM 4-PBA or HLY78 for 48 h. After treatment, mitochondria were extracted using a Mitochondria Isolation Kit (Pierce, Rockford, IL, USA). An equal volume of mitochondria suspension was added into the reaction tube of an oxygen electrode. The oxygen content at different time points was recorded and expressed as a percentage of oxygen consumption per milligram of protein.

Tumor Xenograft In Vivo

Tumor xenograft in nude mice was established using AGS. AGS cells (5×10^6) were transfected with LV-Ctrl or LV-ORP8, which were suspended in 100 ml of a 1:1 mixture of culture medium and growth factor-reduced Matrigel. The cells were implanted subcutaneously into the forelegs of nude mice. Two weeks after implantation, mice injected with LV-ORP8 were then injected with 1 mg/kg 4-PBA or HLY78 into the tumors. The experimental period was 4 weeks, and tumor growth was monitored and weighed. Treatment of the animals was approved by the Animal Care and Use Committee of The First Affiliated Hospital of Xinxiang Medical University and followed the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Quantitative Real-Time PCR

Tissues and cells were lysed with TRIzol (Invitrogen) for total RNA extraction. Total RNA (1 mg) was then subjected to cDNA synthesis with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, P.R. China). Finally, quantitative real-time PCR was performed to analyze relative expression of mRNA levels of target genes with the SYBR Real-Time PCR Kit (TaKaRa). The primers used are listed as follows: β-actin, 5′-GGCA TCCTCACCCTGAAGTA-3′ (forward) and 5′-AGGTGT GGTGCCAGATTTTC-3′ (reverse); ORP8, 5′-ATTagatct ATGAGTCAGCGCCAAGG-3′ (forward) and 5′-AATgtc gacCTACTTGAACATGAAGTTTATTATG-3′ (reverse).

Western Blot Analysis

Tumor tissues were cut, and cells were scraped from the dishes. Tissue pieces and cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 0.1% SDS] with a protease inhibitor cocktail (Roche Diagnostics, Switzerland) on ice for 20 min. The lysates were centrifuged for 20 min at $20{,}000\times g$. Protein concentration was determined using the BCA method (Thermo Fisher Scientific, Waltham, MA, USA), and lysates were mixed with loading buffer. The protein mixtures were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies and then incubated with HRP-conjugated secondary antibody (Thermo Fisher Scientific). Bands were visualized by chemiluminescence reaction using an ECL detection system (Thermo Fisher Scientific), followed by capture using Bio-Rad Imaging Systems (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All assays were carried out in three independent experiments, and the results were expressed as the mean ± SEM and analyzed by GraphPad Prism software (LaJolla, CA, USA). The statistical significance of differences among more than two groups was analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test for multiple comparisons. The statistical significance of differences between two groups was analyzed with two-sided

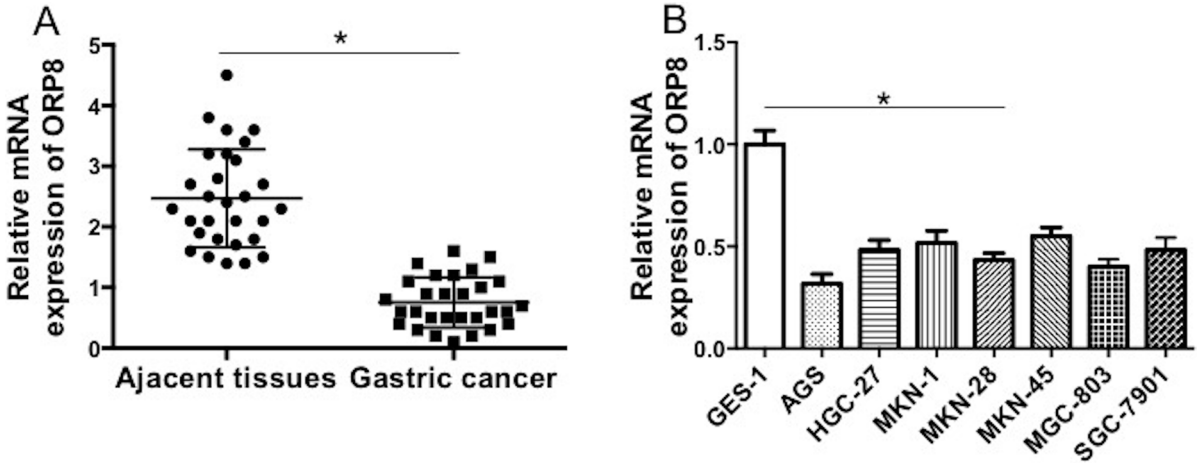

Figure 1. ORP8 expression in GC tissues and cells. mRNA expression of ORP8 in GC and normal adjacent tissues (A) and GES-1 and GC cell lines (B). $*p<0.05$.

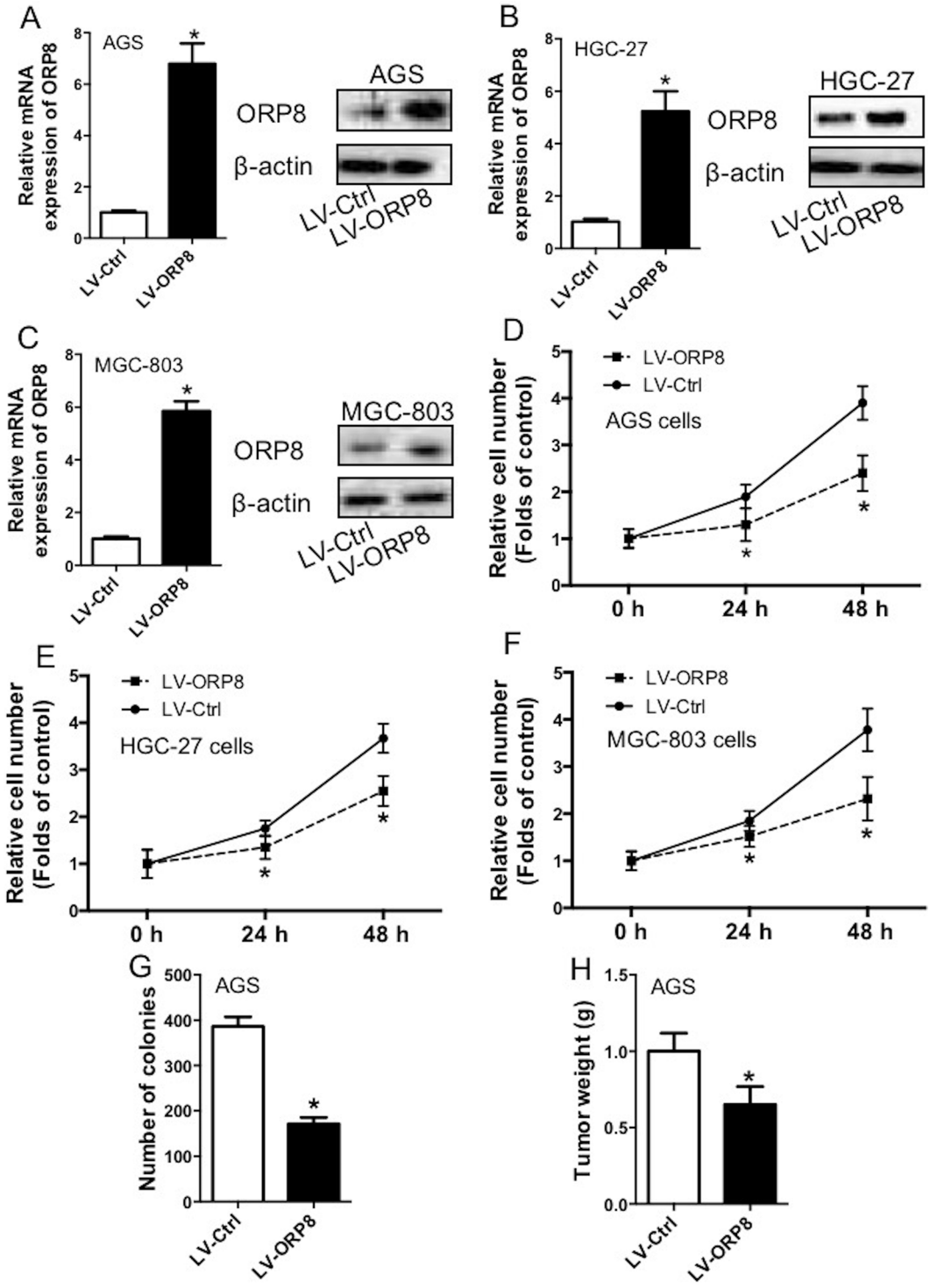

Figure 2. Effect of ORP8 knockup on GC cell growth in vitro and in vivo. AGS, HGC-27, and MGC-803 cells were transfected with LV-Ctrl or LV-ORP8. (A–C) mRNA expression of ORP8. (D–F) Cell proliferation determined by the CCK-8 assay kit. (G) Number of colonies formed in AGS cells. LV-Ctrl or LV-ORP8 AGS cells were implanted in nude mice. (H) Tumor weight in AGS-implanted tumors. *$p < 0.05$, compared with control.

unpaired Student's *t*-tests. The data were deemed to be statistically significant with a value of $p < 0.05$.

RESULTS

Expression Pattern of ORP8 in GC Tissues and Cells

We first detected the expression of ORP8 in GC tissues and cell lines. The mRNA expression of ORP8 in GC tissues was significantly reduced, compared with adjacent tissues (Fig. 1A). Compared with normal human gastric epithelial cell line GES-1, the mRNA expression of ORP8 in GC cells (AGS, HGC-27, MKN-1, MKN-28, MKN-45, MGC-803, and SGC-7901) was significantly lowered (Fig. 1B). These results showed a significantly decreased expression of ORP8 in GC tissues and cells.

ORP8 Knockup Inhibited GC Cell Growth In Vitro and In Vivo

To examine the role of ORP8 in the development of GC, we transfected AGS, HGC-27, and MGC-803 cells with lentivirus (LV) carrying ORP8. The results showed that transfection of ORP8 markedly increased the mRNA and protein expression of ORP8 in AGS, HGC-27, and MGC-803 cells (Fig. 2A–C). We found that knockup of ORP8 notably decreased the proliferation in AGS, HGC-27, and MGC-803 cells (Fig. 2D–F). Overexpression of ORP8 reduced the formation of cellular colonies in AGS cells (Fig. 2G). Moreover, overexpression of ORP8 markedly inhibited the growth of tumor weight in AGS-implanted nude mice (Fig. 2H). The results indicated that ORP8 exhibited an inhibitory role in the development of GC.

ORP8 Knockup Promoted ER Stress in GC Cells In Vitro and In Vivo

To investigate the mechanism of ORP8-induced regulation of GC cell proliferation, ER stress-related factors were evaluated in LV-ORP8-transfected AGS cells and implanted tumors. We showed that knockup of ORP8 significantly increased the expression of CHOP and GRP78

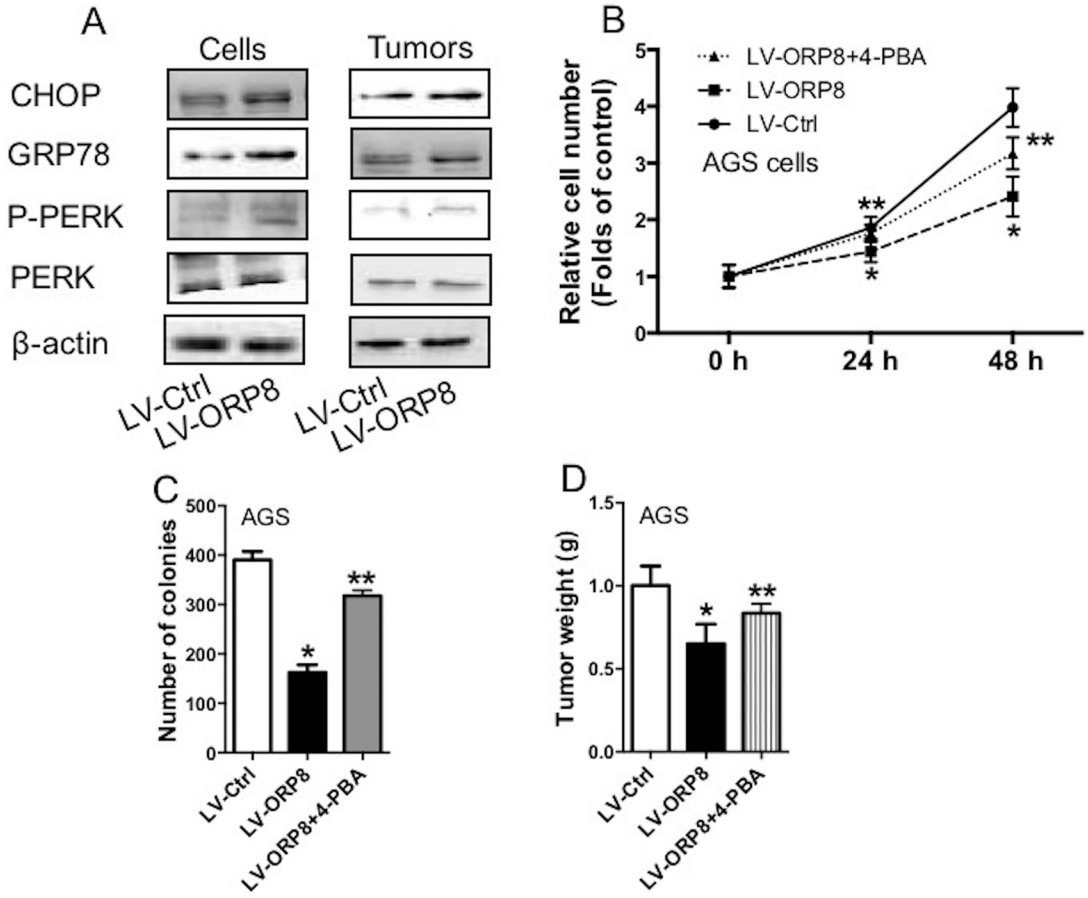

Figure 3. Effect of ORP8 knockup on ER stress in GC cells in vitro and in vivo. AGS was transfected with LV-Ctrl or LV-ORP8. LV-Ctrl or LV-ORP8 AGS cells were implanted in nude mice. (A) Protein expression of CHOP, GRP78, P-PERK, and PERK. AGS cells transfected with LV-ORP8 were treated with 10 μM 4-PBA for 48 h. (B) Cell proliferation determined by the CCK-8 assay kit. (C) Number of colonies formed in AGS cells. Nude mice implanted with LV-ORP8 AGS cells were injected with 1 mg/kg 4-PBA in the tumors 2 weeks after implantation. (D) Tumor weight in AGS-implanted tumors. *$p < 0.05$, compared with control. **$p < 0.05$, compared with ORP8 knockup.

and the phosphorylation of PERK in GC cells and tumors (Fig. 3A), indicating that ER stress occurred upon the upregulation of ORP8. To test the role of ER stress in ORP8-induced regulation of GC cell proliferation, GC cells and tumors were injected with 4-PBA, an inhibitor of ER stress. The results showed that the injection of 4-PBA significantly inhibited overexpression of ORP8-induced decrease in cell proliferation (Fig. 3B), formation of colonies (Fig. 3C), and tumor weight (Fig. 3D), indicating that induction of ER stress was involved in the antitumor role of ORP8 against GC.

ORP8 Knockup Inhibited Wnt Signaling in GC Cells In Vitro and In Vivo

In the next step, we examined the effect of overexpression of ORP8 on Wnt signaling transduction. We found that knockup of ORP8 significantly decreased the protein expression of Wnt3a and β-catenin in GC cells and tumors (Fig. 4A), indicating the inhibition of Wnt signaling induced by the upregulation of ORP8. Moreover, the presence of 4-PBA significantly blocked overexpression of ORP8-induced decrease in Wnt3a and β-catenin, indicating that ER stress was involved in the inhibition of Wnt signaling (Fig. 4A). To test whether the inhibition of Wnt signaling was involved in the overexpression of ORP8-induced decrease in GC cell proliferation, cells were treated with HLY78, an activator of Wnt signaling. We showed that HLY78 significantly suppressed ORP8-induced decrease in GC cell proliferation (Fig. 4B), formation of colonies (Fig. 4C), and tumor weight (Fig. 4D). These results indicated that ER stress-mediated inhibition of Wnt signaling was involved in the antitumor role of ORP8 against GC.

ORP8 Knockup Activated Mitochondrial Apoptotic Cell Death in GC Cells In Vitro and In Vivo

Furthermore, we tested the effect of overexpression of ORP8 on apoptotic cell death. We showed that overexpression of ORP8 resulted in a significant increase in apoptotic cell death in AGS cells (Fig. 5A and B). Incubation with 4-PBA and HLY78 significantly suppressed overexpression of ORP8-induced increase in

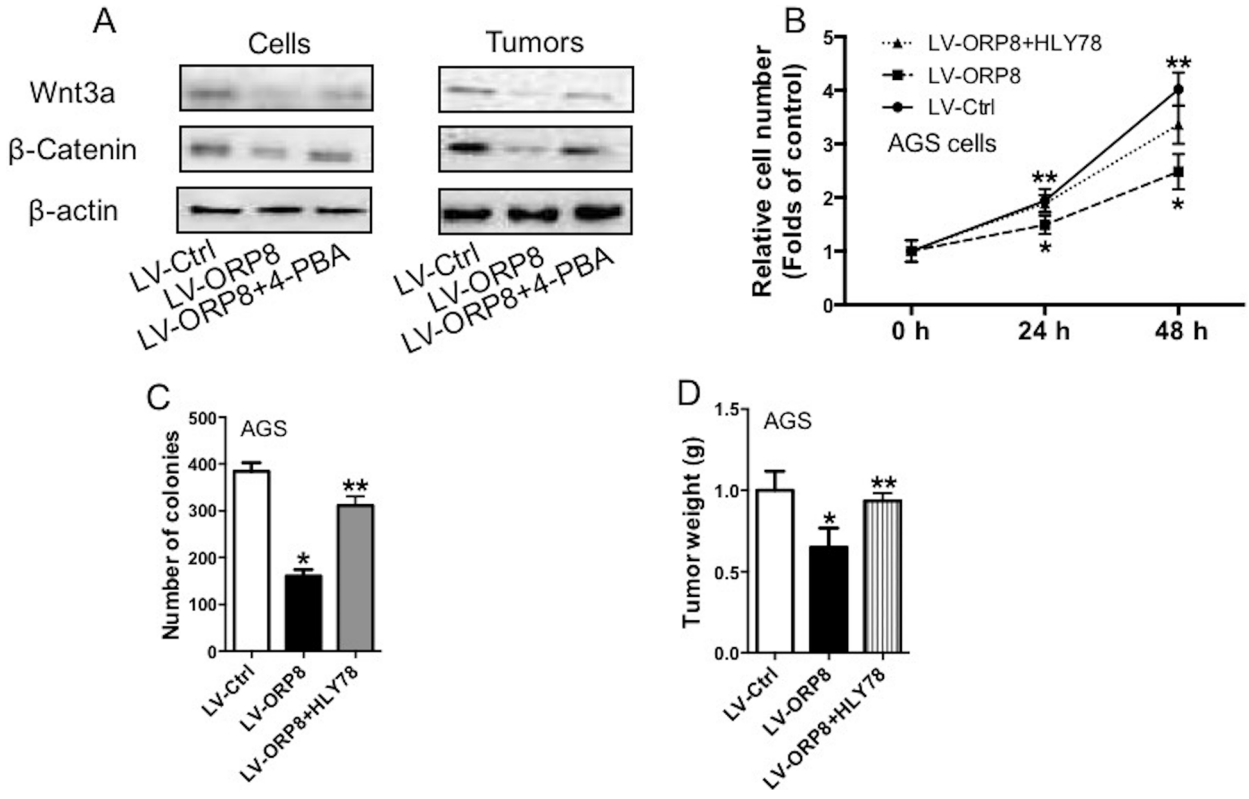

Figure 4. Effect of ORP8 knockup on Wnt signaling in GC cells in vitro and in vivo. AGS was transfected with LV-Ctrl or LV-ORP8. LV-Ctrl or LV-ORP8 AGS cells were implanted in nude mice. (A) Protein expression of Wnt3a and β-catenin. AGS cells transfected with LV-ORP8 were treated with 10 μM HLY78 for 48 h. (B) Cell proliferation determined by the CCK-8 assay kit. (C) Number of colonies formed in AGS cells. Nude mice implanted with LV-ORP8 AGS cells were injected with 1 mg/kg HLY78 in the tumors 2 weeks after the implantation. (D) Tumor weight in AGS-implanted tumors. *$p<0.05$, compared with control. **$p<0.05$, compared with ORP8 knockup.

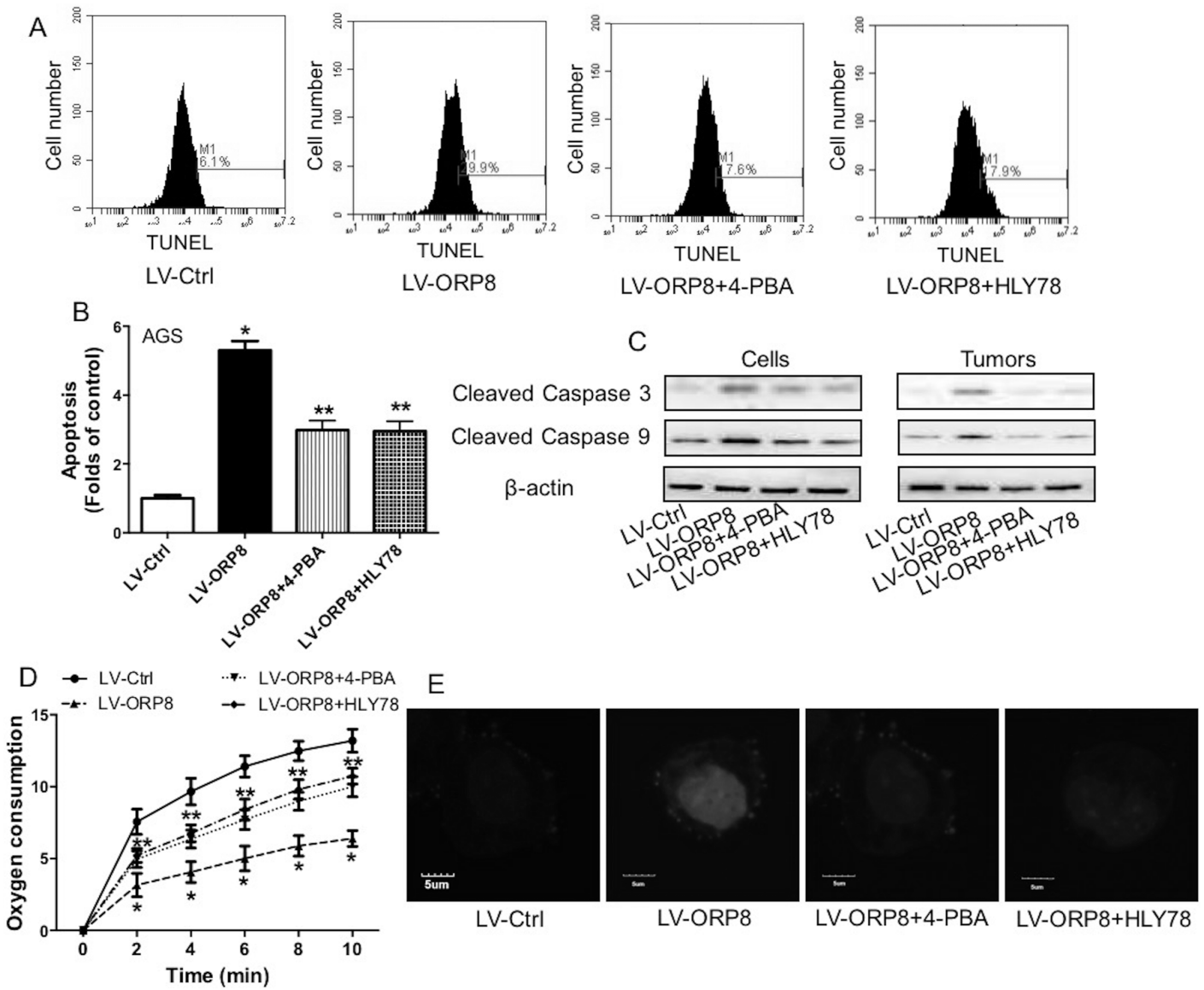

Figure 5. Effect of ORP8 knockup on mitochondrial apoptosis in GC cells in vitro and in vivo. AGS was transfected with LV-Ctrl or LV-ORP8 and treated with or without 10 μM HLY78 for 48 h. LV-Ctrl or LV-ORP8 AGS cells were implanted in nude mice with or without injection of 1 mg/kg HLY78 in the tumors 2 weeks after implantation. (A) Apoptotic cell death analyzed by flow cytometry. (B) Statistical analysis of apoptotic cell death. (C) Protein expression of cleaved caspase 3 and caspase 9. (D) Mitochondrial oxygen consumption rate. (E) ROS level in cells stained with DHE. *$p<0.05$, compared with control. **$p<0.05$, compared with ORP8 knockup.

apoptosis in AGS cells (Fig. 5A and B). Overexpression of ORP8 induced a significant increase in the protein expression of cleaved caspase 3 and caspase 9 in cells and tumors (Fig. 5C). Treatment with 4-PBA and HLY78 significantly suppressed overexpression of ORP8-induced increase in the expression of cleaved caspase 3 and caspase 9 (Fig. 5C). Moreover, overexpression of ORP8 resulted in a significant decrease in mitochondrial oxygen consumption, which was inhibited by 4-PBA and HLY78 (Fig. 5D). Furthermore, overexpression of ORP8 markedly increased MitoSOX staining in AGS cells, indicating the increase in mitochondrial ROS level (Fig. 5E). Treatment with 4-PBA and HLY78 significantly inhibited the increase in ROS level. These results indicated that mitochondrial apoptosis mediated by ER stress and inhibition of Wnt signaling was involved in the antitumor role of ORP8 against GC (Fig. 6).

DISCUSSION

Previous literature shows that ORP8 has the potential to induce cytotoxicity in hepatocellular carcinoma cells[22] and increase the sensitivity to Fas-mediated apoptosis[21]. The findings suggest a potential antitumor role for ORP8. The present study was designed to investigate the potential role of ORP8 in the development of GC. We found that ORP8 expression was lower in GC tissues and cells. Overexpression of ORP8 significantly inhibited cell proliferation in several GC cell lines, suppressed the formation of colonies in AGS cells, and decreased tumor weight implanted in nude mice. These results demonstrate that ORP8 is a negative regulator of GC tumor growth.

The normal function of ER is critical for multiple cellular activities and cell survival[23–25]. Abnormal function of ER results in the accumulation of unfolded proteins and the activation of unfolded protein response, which is harmful to cell growth[23–25]. In particular, ER stress may activate cell death in cancer and is usually involved in the antitumor effect of various cytotoxic agents[26,27]. ORP8 plays a role in the transportation of phosphatidylserine to ER–plasma membrane contact sites[20], indicating a potential role in the regulation of ER function. We found that overexpression of ORP8 induced significant ER stress, as reflected by increased expression of CHOP, GRP78, and P-PERK. Moreover, inhibition of ER stress by 4-PBA significantly blocked the decrease in cell proliferation, colony formation, and tumor weight induced by ORP8. The results suggest that ER stress is involved in the inhibitory role of ORP8 in the development of GC.

The pathophysiological process of ER stress is closely interacted with Wnt signaling in the regulation of cell growth and death[28–30]. Abnormal Wnt signaling exists in tumor development[31]. In the current study, we found that overexpression of ORP8 resulted in a significant decrease in Wnt3a/β-catenin expression, indicating inhibition of Wnt signaling. Suppression of ER stress significantly blocked overexpression of ORP8-induced decrease in Wnt3a/β-catenin expression. Activation of Wnt signaling could prohibit overexpression of ORP8-induced decrease in cell proliferation and tumor growth. These findings suggest that overexpression of ORP8-induced inhibition of Wnt signaling is mediated by ER stress, and the reduction of Wnt signaling contributed to the antitumor role of ORP8 in GC.

Tumor growth is usually controlled by the interaction between cell proliferation and cell death[32–35]. ER stress could induce mitochondrial apoptotic cell death through direct interaction and regulation of mitochondrial apoptotic events[32–35]. Activation of Wnt signaling is associated with the proliferation of several cancers, and reduction of Wnt signaling is related to apoptosis[36,37]. In this study,

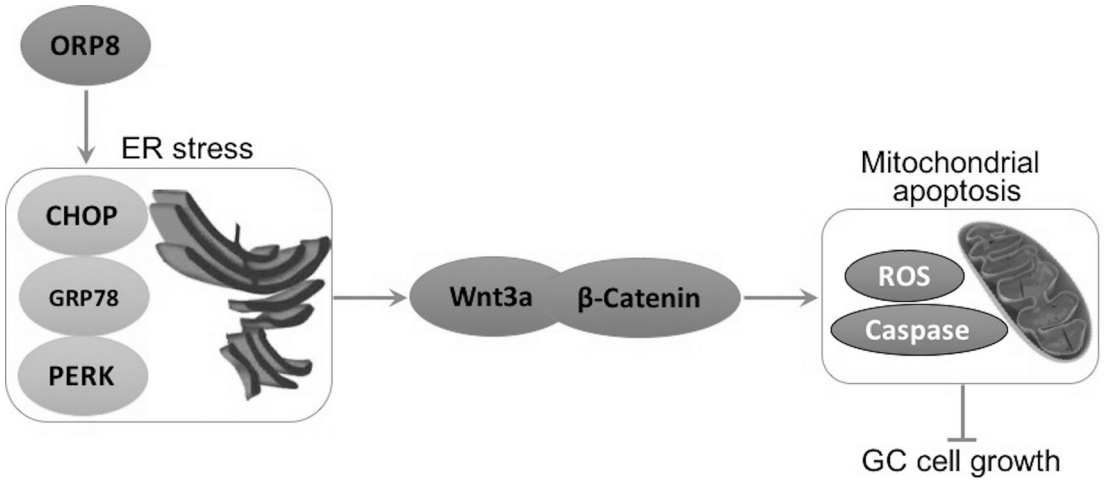

Figure 6. Mechanism of the antitumor role of ORP8 in GC.

we showed that overexpression of ORP8 results in a significant increase in mitochondrial ROS, a decrease in mitochondrial oxygen consumption, activation of mitochondrial caspase cascades, and a significant increase in apoptotic cell death. Inhibition of ER stress and activation of Wnt signaling could attenuate overexpression of ORP8-induced mitochondrial apoptotic signaling and apoptosis. These results indicate that ER stress, attenuation of Wnt signaling, and the subsequent apoptosis are involved in the antitumor role of ORP8 in GC.

In conclusion, we found that overexpression of ORP8 significantly inhibits GC cell proliferation and implanted tumor growth in vivo. Induction of ER stress, inhibition of Wnt signaling, and apoptotic cell death are involved in ORP8-induced inhibition of GC cell proliferation. These findings indicate that downregulation of ORP8 plays a pivotal role in the progression of GC, and it may be a novel therapeutic target for the treatment of GC.

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