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Original Article

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HSPA8-mediated stability of the CLPP protein regulates mitochondrial autophagy in cisplatin-resistant ovarian cancer cells

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Abstract

Currently, platinum agents remain the mainstay of chemotherapy for ovarian cancer (OC). However, cisplatin (DDP) resistance is a major reason for chemotherapy failure. Thus, it is extremely important to elucidate the mechanism of resistance to DDP. Here, we establish two DDP-resistant ovarian cancer cell lines and find that caseinolytic protease P (CLPP) level is significantly downregulated in DDP-resistant cell lines compared to wild-type ovarian cancer cell lines (SK-OV-3 and OVcar3). Next, we investigate the functions of CLPP in DDP-resistant and wild-type ovarian cancer cells using various assays, including cell counting kit-8 assay, western blot analysis, immunofluorescence staining, and detection of reactive oxygen species (ROS) and apoptosis. Our results show that *CLPP* knockdown significantly increases the half maximal inhibitory concentration (IC_{50}) and mitophagy of wild-type SK-OV-3 and OVcar3 cells, while CLPP overexpression reduces the IC_{50} values and mitophagy of DDP-resistant SK-OV-3 and OVcar3 cells. Next, we perform database predictions and confirmation experiments, which show that heat shock protein family A member 8 (HSPA8) regulates CLPP protein stability. The dynamic effects of the HSPA8/CLPP axis in ovarian cancer cells are also examined. HSPA8 increases mitophagy and the IC_{50} values of SK-OV-3 and OVcar3 cells but inhibits their ROS production and apoptosis. In addition, CLPP partly reverses the effects induced by HSPA8 in SK-OV-3 and OVcar3 cells. In conclusion, CLPP increases DDP resistance in ovarian cancer by inhibiting mitophagy and promoting cellular stress. Meanwhile, HSPA8 promotes the degradation of CLPP protein by regulating its stability.

Key words cisplatin resistance, ovarian cancer, caseinolytic protease P, heat shock protein family A member 8, mitophagy

Introduction

Ovarian cancer is the 3rd most common gynecologic malignancy worldwide and the most lethal gynecologic malignancy [1]. Currently, the incidence and mortality rates of ovarian cancer continue to increase [2]. The mainstream chemotherapy for epithelial ovarian cancer (EOC) is a platinum agent combined with taxane [3]. However, patients eventually develop tolerance to cisplatin (DDP)-based therapy after a few cycles of treatment, which further increases the mortality rate [4]. Thus, there is an urgent need to elucidate the underlying mechanism of DDP resistance in ovarian cancer.

DDP acts by binding to DNA and subsequently causing DNA damage, inhibition of DNA replication, and induction of cell apoptosis [5]. Based on DDP pharmacological studies and its biological activities, various mechanisms have been proposed to contribute to DDP resistance, including an increased cellular efflux of drugs [6], reduced drug influx [7], inhibition of apoptosis [8], dysregulation of DNA damage repair systems [9], enhanced activity of drug-metabolizing enzymes [10,11], and an altered cellular microenvironment [12–14]. The reasons for DDP resistance in DDP-resistant cancer cells include a reduction in drug accumulation, DDP inactivation resulted from its reaction with glutathione and metallothionein, and a rapid repair of DNA lesions [9,15]. Copper efflux transporters, such as Cu-transporting P-type ATPases (ATP7A and ATP7B), have been shown to regulate the efflux of DDP [16,17]. Recent studies have shown that changes in mitochondrial function and abnormal autophagy play key roles in DDP resistance in ovarian cancer [18–20], which suggests that

mitochondrial molecules are involved in DDP resistance.

Mitochondrial caseinolytic protease P (CLPP) is a serine protease located in the mitochondrial matrix and is involved in mitochondrial protein metabolism (proteostasis) and oxidative stress by facilitating the degradation of misfolded or damaged proteins and thus the maintenance of protein metabolism homeostasis [21,22]. Due to the functions of CLPP in mitochondria, it has multiple effects on tumors [23]. In acute myeloid leukemia, the genetic and chemical activation of CLPP selectively kills cancer cells by degrading respiratory chain protein substrates and disrupting mitochondrial structure and function; however, CLPP has no effects on noncancerous cells [24]. In pancreatic ductal adenocarcinoma (PDAC), the chemical activation of CLPP increases the degradation of respiratory chain complexes, which causes an endoplasmic reticulum stress response that suppresses the growth of PDAC cells [25]. In glioblastoma, CLPP activators induce synthetic lethality by inhibiting oxidative energy metabolism and reducing cell viability [26]. In EOC, the mitochondrial deficits induced by CLPP inhibit the growth and metastasis of EOC cells [27]. However, the role of CLPP in DDP resistance in ovarian cancer remains unclear.

Heat shock protein family A member 8 (HSPA8) belongs to the heat shock protein 70 (HSP70) family [28]. It plays important physiological roles in protein metabolism and homeostasis, both of which depend on constant protein degradation and resynthesis [29]. Under normal or stressful conditions, eukaryotic cells remove misfolded proteins via autophagy. In chaperone-mediated autophagy, HSPA8 recognizes and targets cytosolic proteins with a signature exposed pentapeptide motif (KFERQ) [30]. After being recognized by HSPA8 and binding to lysosomal-associated membrane protein 2A, the target proteins are translocated into the lysosomal lumen for degradation [31].

In this study, we explored the effects of CLPP on mitophagy in wild-type or DDP-resistant ovarian cancer cells. Specifically, our database predictions and subsequent experiments indicated that the *HSPA8* gene might regulate the stability of CLPP. Taken together, our findings suggest that HSPA8-mediated stability of the CLPP protein regulates mitochondrial autophagy in DDP-resistant ovarian cancer cells. Our results elucidate one of the mechanisms by which drug resistance occurs, provide new insights into cisplatin resistance in ovarian cancer, and will facilitate the development of new clinical therapies for ovarian cancer.

Materials and Methods

Cell lines and cell transfection

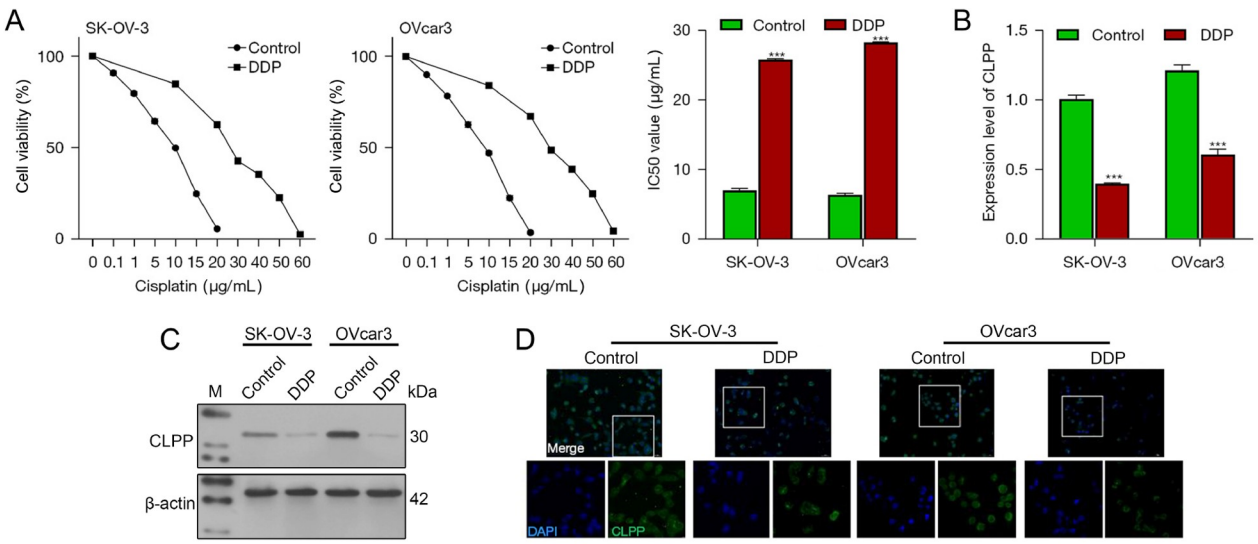

Figure 1. CLPP expression is upregulated in DDP-resistant ovarian cancer cells (A) SK-OV-3 and OVcar3 cells were treated with DDP (0, 0.1, 1, 5, 10, 15, 20, 30, 40, 50, or 60 μg/mL) and examined for cell viability by the CCK-8 assay. DDP-resistant ovarian cancer cells were established. (B) The levels of CLPP mRNA in wild-type and DDP-resistant ovarian cancer cells were examined by real-time PCR. (C) The levels of CLPP protein in the cells were examined by western blot analysis. (D) The distribution of CLPP protein in the cells was examined by immunofluorescence staining (magnification 200 × and 400 ×). ***$P < 0.001$ compared to the control group. CLPP, caseinolytic protease P; DDP, cisplatin; CCK-8, cell counting kit-8; PCR, polymerase chain reaction. Control, wild-type SK-OV-3 and OVcar3 cell lines.

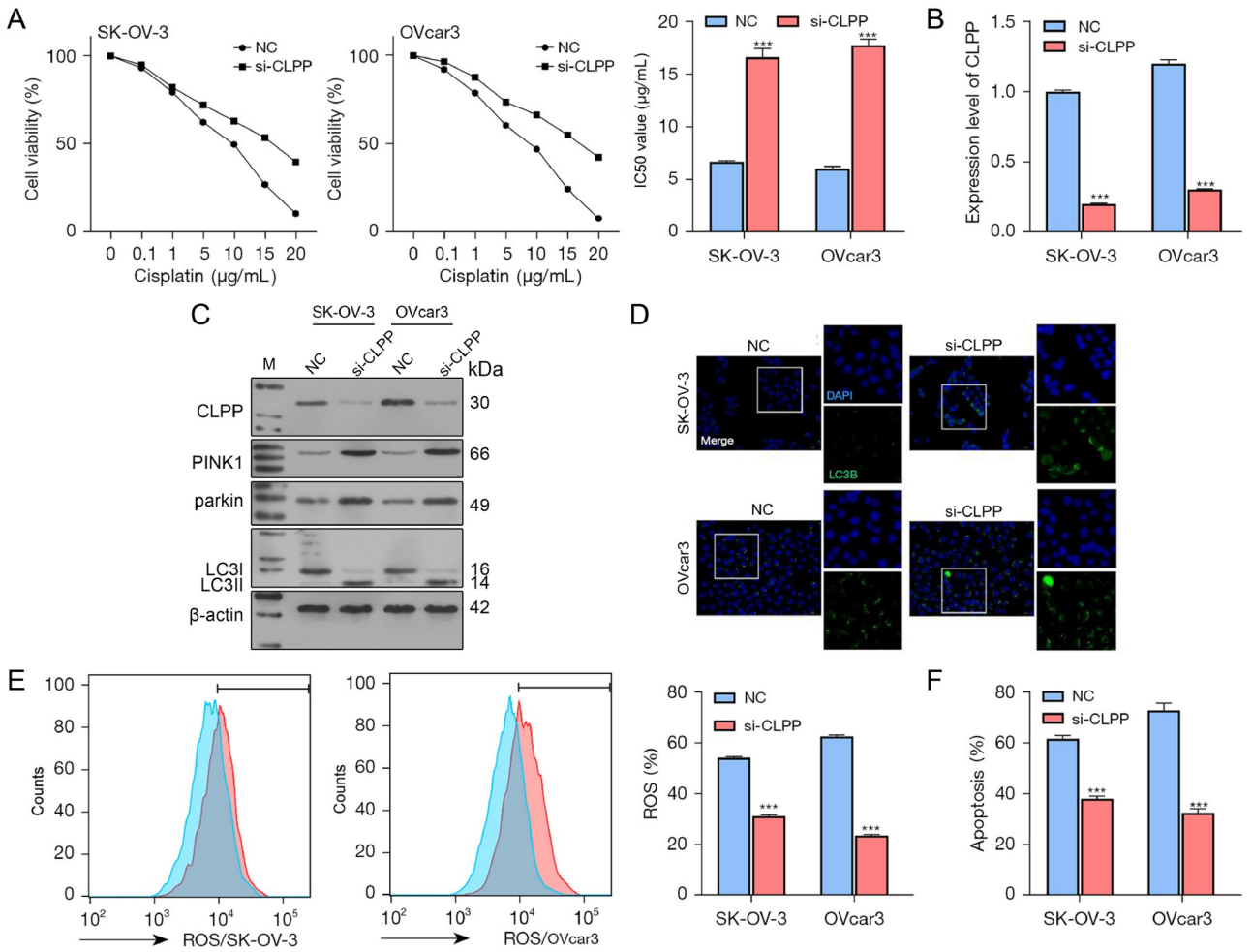

Figure 2. The effects of *CLPP* knockdown on wild-type ovarian cancer cells (A) Wild-type ovarian cancer cells were transfected with a CLPP interference sequence and then treated with different concentrations of DDP; the IC_{50} values were calculated based on cell viability. (B) The levels of CLPP mRNA in the cells were examined by real-time PCR. (C) The protein levels of CLPP, PINK1, Parkin, and LC3II/I were examined by western blot analysis. (D) The distribution of LC3B protein in the cells was examined by immunofluorescence staining (magnification 200 × and 400 ×). (E) ROS levels were measured by flow cytometry. (F) Cell apoptosis was examined by flow cytometry. ***$P < 0.001$ compared to the NC group. NC, negative control; CLPP, caseinolytic protease P; DDP, cisplatin; ROS, reactive oxygen species; si, small interfering; PCR, polymerase chain reaction.

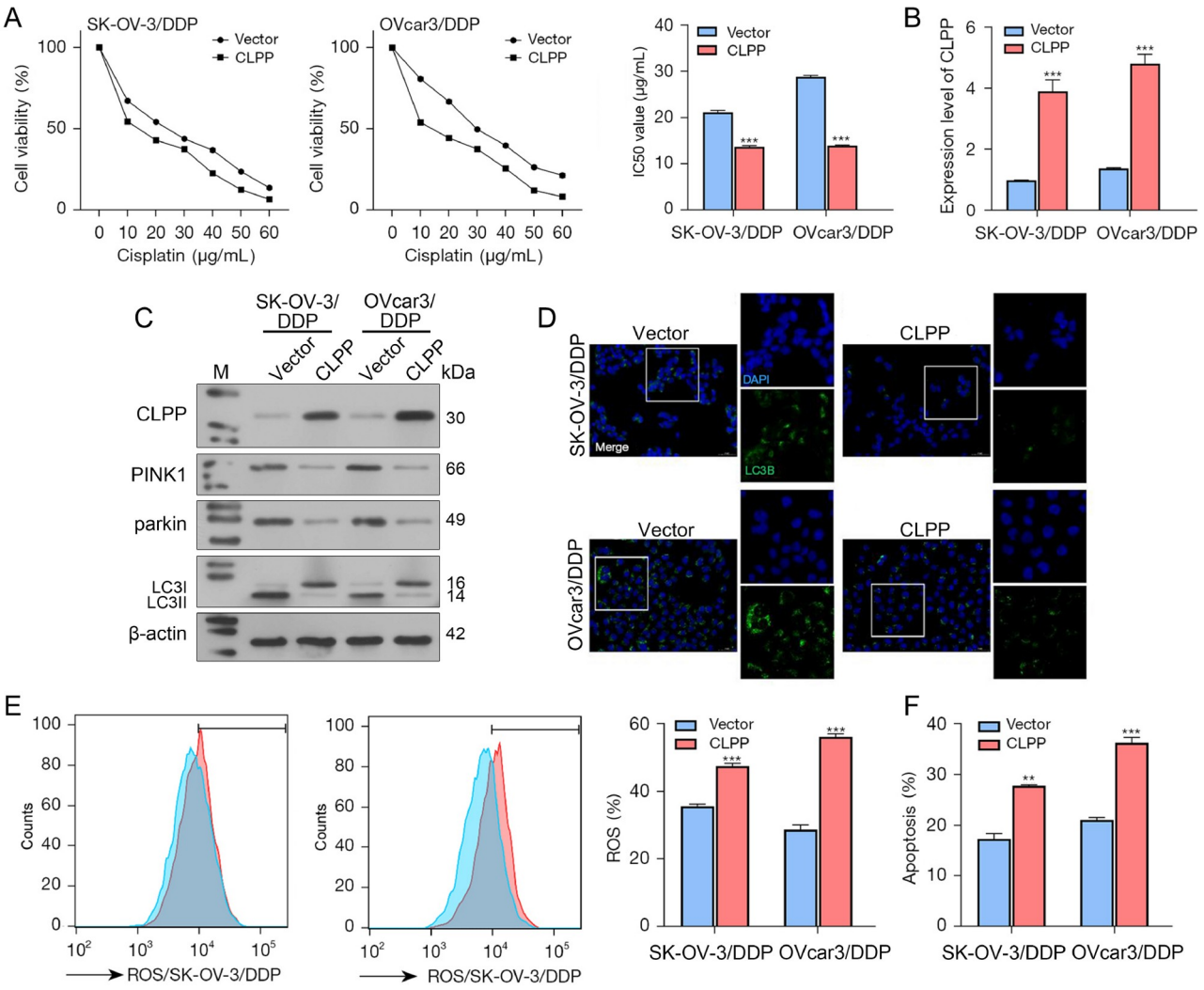

Figure 3. The effects of CLPP overexpression on DDP-resistant ovarian cancer cells (A) DDP-resistant ovarian cancer cells were transfected with CLPP overexpression plasmids and subsequently treated with different concentrations of DDP; the IC_{50} values were calculated based on cell viability. (B) The levels of CLPP mRNA in the cells were examined by real-time PCR. (C) The protein levels of CLPP, PINK1, Parkin, and LC3II/I were examined by western blot analysis. (D) The distribution of LC3B protein in the cells was examined by immunofluorescence staining (magnification 200× and 400×). (E) ROS levels were measured by flow cytometry. (F) Cell apoptosis was examined by flow cytometry. $^{**}P<0.01$, $^{***}P<0.001$ compared to the vector group. Vector, empty carrier; CLPP, caseinolytic protease P; DDP, cisplatin; ROS, reactive oxygen species; si, small interfering; PCR, polymerase chain reaction.

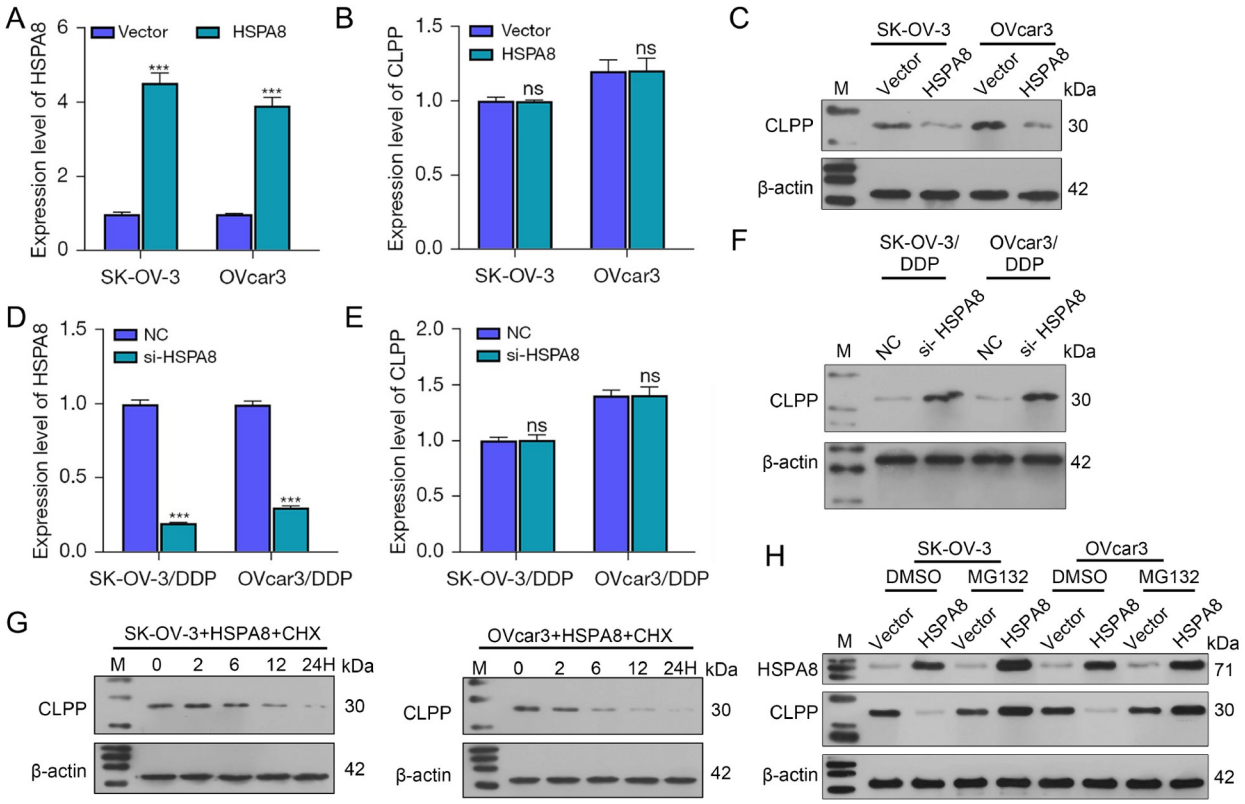

Figure 4. HSPA8 affects CLPP protein stability in ovarian cancer cells (A) HSPA8 overexpression was established in SK-OV-3 and OVcar3 cells as confirmed by real-time PCR. (B) The levels of CLPP mRNA in the transfected SK-OV-3 and OVcar3 cells were determined by real-time PCR. (C) The levels of CLPP protein in the transfected SK-OV-3 and OVcar3 cells were determined by western blot analysis. (D) *HSPA8* knockdown was established in DDP-resistant SK-OV-3 and OVcar3 cells as confirmed by real-time PCR. (E) The levels of CLPP mRNA in the transfected DDP-resistant SK-OV-3 and OVcar3 cells were determined by real-time PCR. (F) The levels of CLPP protein in the transfected DDP-resistant SK-OV-3 and OVcar3 cells were determined by western blot analysis. (G) The levels of CLPP protein in HSPA8-overexpressing SK-OV-3 and OVcar3 cells treated with CHX were determined by western blot analysis. (H) SK-OV-3 and OVcar3 cells with stable overexpression of HSPA8 were treated with a proteasome inhibitor (MG132, 20 μM). The levels of HSPA8 and CLPP proteins were detected by western blot analysis. ***$P < 0.001$ compared to the vector or NC group. Vector, empty carrier; NC, negative control; CLPP, caseinolytic protease P; CHX, cycloheximide; DDP, cisplatin; HSPA8, heat shock protein family A member 8; M, marker; ns, not significant; PCR, polymerase chain reaction; si, small interfering.

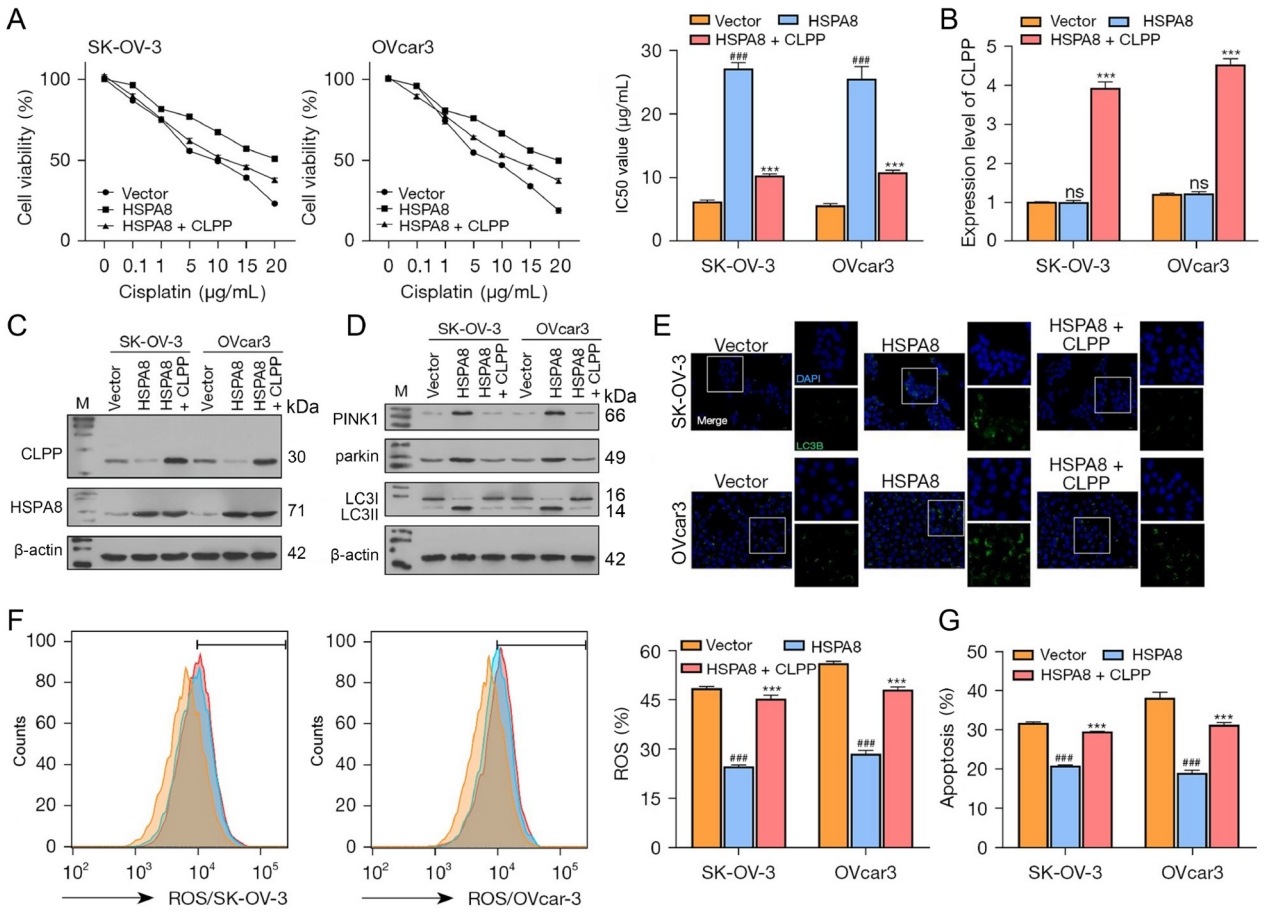

Figure 5. HSPA8 affects wild-type ovarian cancer cell phenotypes by downregulating CLPP expression (A) Wild-type ovarian cancer cells were cotransfected with HSPA8 and CLPP overexpression plasmids and then treated with different concentrations of DDP; the IC_{50} values were calculated based on cell viability. (B) The levels of CLPP mRNA in the cells were examined by real-time PCR. (C,D) The levels of CLPP, PINK1, Parkin, and LC3II/I proteins were examined by western blot analysis. (E) The distribution of LC3B protein in the cells was examined by immunofluorescence staining (magnification 200× and 400×). (F) ROS production was measured by flow cytometry. (G) Cell apoptosis was examined by flow cytometry. $^{\#\#\#}P < 0.001$ compared to the vector group; $^{***}P < 0.001$ compared to the HSPA8 group. CLPP, caseinolytic protease P; DDP, cisplatin; HSPA8, heat shock protein family A member 8; M, marker; ns, not significant; PCR, polymerase chain reaction; ROS, reactive oxygen species.

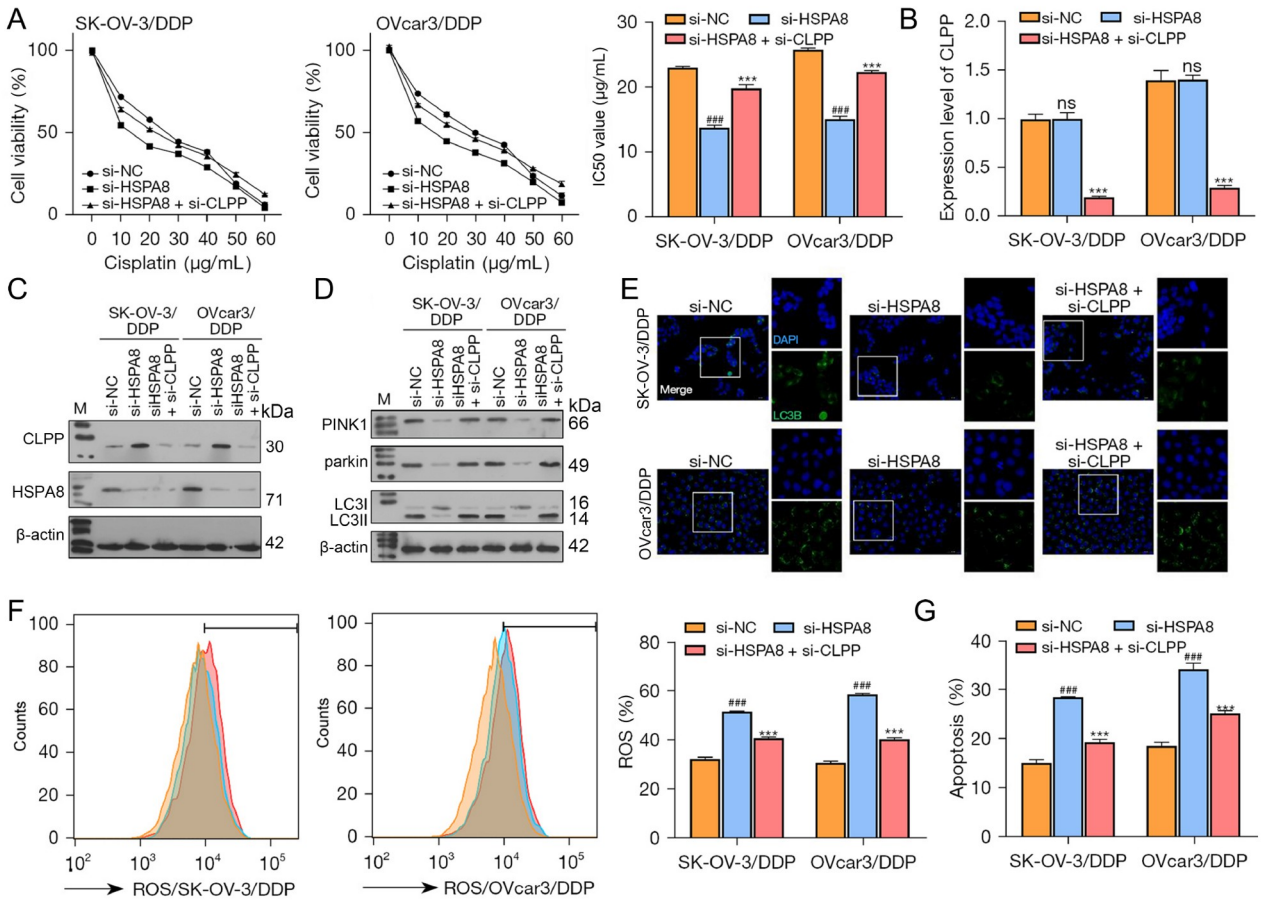

Figure 6. ***HSPA8*** **knockdown upregulates CLPP expression to affect DDP-resistant ovarian cancer cell phenotypes** (A) DDP-resistant ovarian cancer cells were transfected with HSPA8 and the CLPP interference sequence and then treated with different concentrations of DDP; the IC_{50} values were calculated based on cell viability. (B) The levels of CLPP mRNA in the cells were examined by real-time PCR. (C, D) The protein levels of CLPP, HSPA8, PINK1, Parkin, and LC3II/I were examined by western blot analysis. (E) The distribution of LC3B protein in cells was examined by immunofluorescence staining (magnification, 200× and 400×). (F) ROS production was measured by flow cytometry. (G) Cell apoptosis was examined by flow cytometry. ###$P < 0.001$ compared to the si-NC group; ***$P < 0.001$ compared to the si-HSPA8 group. si-NC, negative control; CLPP, caseinolytic protease P; DDP, cisplatin; HSPA8, heat shock protein family A member 8; M, marker; ns, not significant; PCR, polymerase chain reaction; ROS, reactive oxygen species; si, small interfering.