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Original Article

Potential role of HGF-PARP-1 signaling in invasion of ovarian cancer cells

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Abstract: We investigated the effects and signaling pathways involved in both HGF-mediated regulation of PARP-1 expression and the invasion ability of ovarian cancer cells. Using a transwell assay, the invasiveness of SKOV-3 cells was tested by incubating them with increasing concentrations of HGF. The relative expression levels of PARP-1 after HGF treatment were analyzed by Real-Time PCR and western blotting. SKOV3 cells were transfected with either negative control siRNA or PARP-1 siRNA, and were divided into different groups as follows: control group; HGF group; PARP-siRNA group; HGF+PARP-siRNA group; NC-siRNA group; and HGF+NC-siRNA group. Western blotting was employed to measure the expression of PARP-1 in the different groups. Transwell tests were used to examine invasiveness. ELISA was applied to measure MMP-2 expression. HGF promotes cell invasion in a concentration- and time-dependent manner in SKOV-3 cells. The expression levels of PARP-1 increased after administration of 40 ng/ml HGF for 24 h. The expression of PARP-1 in the PARP1-siRNA group was lower compared with that in the NC-siRNA group ($P < 0.05$); PARP1-siRNA transfection significantly reduced the impact of HGF on invasiveness and MMP-2 expression in SKOV-3 cells. HGF promotes the invasiveness and metastasis of ovarian cancer cells. This effect could be related to the induction of increased expression levels of MMP-2 mediated by PARP-1.

Keywords: Hepatocyte growth factor (HGF), ovarian cancer, invasion, poly (ADP-ribose) polymerase-1

Introduction

Ovarian cancer is the leading cause of death among all gynecological malignancies [1]. There is a lack of specific symptoms and signs in its early phase. Therefore, it is important to understand the biological behavior of ovarian cancer, to improve the clinical outcome of late-stage patients. However, mechanisms of invasion and metastasis in ovarian cancer remain poorly understood.

Poly ADP-ribose polymerase-1 (PARP-1) is a protein post-translational modification enzyme that is present in most eukaryotic cells and plays an important role in processes that include DNA repair, cell proliferation, and apoptosis [2]. Recent studies have shown that PARP-1 was multifunctional and had key roles in tumorigenesis, and invasiveness The inhibition of PARP-1 expression can reduce cell proliferation and invasiveness [3, 4]. Cellular expression of PARP-1 is regulated by various mechanisms, such as growth factors, oncogenes, and tumor suppressor genes. Among these growth factors, hepatocyte growth factor (HGF) has been known to play a predominant role in many types of human cancers [5]. HGF, one of the most well-characterized growth factors, carries out an important biological function in promoting processes such as cell division, proliferation, differentiation, morphogenesis, and invasion [6]. Previously, we have detected a positive correlation between HGF and the expression levels of PARP-1 in ovarian cancer, suggesting that these two factors might both contribute to the regulatory mechanism, although their specific roles remain unclear.

Currently, there are limited data available about the relationship between HGF and PARP-1 in ovarian cancer and the exact mechanism of their biological behavior in ovarian cancer remains undefined. This present study was designed to investigate the effects of HGF on the expression levels of PARP-1 in SKOV-3 cells

and the role of the HGF/PARP-1 pathway in regulating the invasion and metastasis of SKOV-3 cells. This research could provide valuable insights into the mechanisms of HGF/PARP-1 signaling pathway in the malignant progression of ovarian cancer and provide important information for development of targeted therapy.

Materials and methods

Cell culture

Human ovarian cancer cells (SKOV3) were purchased from ATCC (Rockville, MD). The SKOV3 cells was cultured in RPMI-1640 medium (Hyclone, Logan, UT) containing 10% FBS at 37°C under an atmosphere of 5% CO_2. Human recombinant HGF was from PeproTech (Rocky Hill, NJ). The cells were harvested at logarithmic growth phase for experiments and cells were divided into following groups: control group (without HGF treatment), HGF treated groups (final concentration at 10, 20, 40, 60, 80 ng/ml). The cells were treated for 6, 24, 48 h respectively.

Transwell assay

The upper portion of Transwell chambers (Corning, NY, USA) were coated with 75 μL of Matrigel (BD Biosciences, Bedford, MA) diluted 1:3 in serum-free medium and incubated at 37°C for 2 h. SKOV3 cells in the logarithmic growth phase were harvested and cultured in serum-free medium at 4×10^5 cells/ml in a single-cell suspension. Complete medium containing 20% FBS was added to 24-well cell culture plates; HGF was added to the experimental group at a final concentration of 10, 20, 40, 60, or 80 ng/ml. Then, transwell chambers were inserted into the 24-well plates, and 200 μl serum-free single cell suspension was added to the upper chamber. Transwell plates were incubated for 24 h with 5% CO_2 in a 37°C incubator. After removing the upper chambers, the lower chambers were fixed in methanol solution for 15 min, and then stained with hematoxylin & eosin (H&E). A total of five fields were randomly selected to count the number of cells at high magnification, and three separate wells were analyzed for each group.

Screening of lentivirus-transfected SKOV3 cells and stable strains

NC-siRNA and PARP1-siRNA lentivectors were constructed by Genechem (Shanghai, China). SKOV3 cells were seeded into 12-well plates at 5×10^4 cells/well. After 24 h culturing, the media was changed with 500 μL viral supernatant containing 5 μg/mL Polybrene, Enhanced Infection Solution. After 16 h culturing, the transfection media were discharged and replaced with normal media for further culturing for 72 h. The transfection efficiency was determined by GFP expression. After transfection for 48 h in the experimental group, the cells were treated with 2 μg/ml puromycin and the medium were changed with fresh medium containing 2 μg/ml puromycin ever 2-3 days. 10 days after screening, the puromycin-resistant clones were amplified and obtained the stable transfected cell line. The silencing effect of PARP-1 was determined by RT-PCR.

Real-time PCR analysis

Total RNA were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated water. Ten microliters of total RNA obtained were converted into cDNA by using oligo-dT15 primer and M-MLV reverse transcriptase (Promega, Madison, WI). RNA transcripts were measured by qRT-PCR, based on general fluorescence detection with SYBR Green (Toyobo, Osaka, Japan), using the Lightcycler (Lightcycler 2.0, Roche, USA). To internally normalize the levels of total RNA present in each reaction, we amplified the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. The real-time PCR reaction conditions were 5 min at 95°C followed by 45 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. Measurements were performed in triplicate. The relative amount of mRNA was calculated as the ratio between the target mRNA and the corresponding endogenous control GAPDH. Gene-specific primers used were as follows, GADPH forward: 5'-CCCTCAAGATCATCAGCAAT-3', reverse: 5'-CCATCCACAGTCTTCTGGGT-3'; and PARP-1 forward: 5'-GCCCTAAAGGCTCAGAACGAC-3', reverse: 5'-CACCATGCCATCAGCTACTCG-3'.

Western blot analysis

Cells were seeded and treated with various concentrations of HGF. Total cellular protein was extracted and protein concentrations were determined using a colorimetric protein assay (Bio-Rad, Cambridge, MA). Equal amounts of

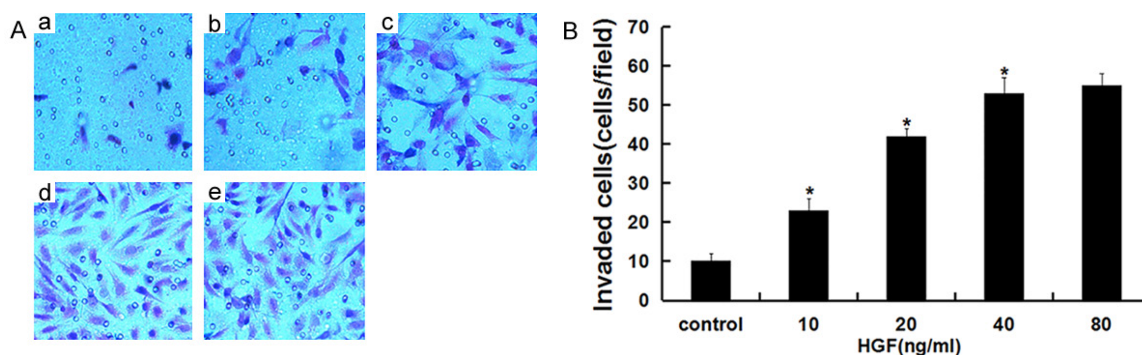

Figure 1. HGF promotes cell invasion in SKOV-3 cells. SKOV-3 cells were incubated with increasing concentrations of HGF to examine the invasive abilities. The number of invasive cells was counted in 5 randomly selected fields. A. Micrographs depicting a random view field on the lower side of the transwell chambers under different experimental conditions (100×). B. Graphs show the dose response effect of HGF; **P* < 0.05 vs. control.

total cellular protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Pierce, Rockford, IL). Membranes were incubated for 2 h in TBS containing 0.1% Tween-20 and 5% bovine serum albumin to block nonspecific binding. Membranes were incubated overnight with primary antibodies at 4°C and then with the appropriate secondary antibodies for 2 h at room temperature. Primary antibodies used were against human PARP-1 (Cell Signaling Technology, Danvers, MA). Signals were detected by an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL), and protein expressions were quantified using the Gel-Pro Analyzer software. Results were then normalized to actin content in the samples.

ELISA analysis

Detection of human MMP-2 in culture supernatants was performed using a specific MMP-2 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The media of SKOV-3 cells under different treatment conditions was harvested and centrifuged. At least three separate supernatant preparations were analyzed. The MMP-2 concentration was determined by interpolating the sample's optical density at 450 nm into the linear range of the standard curve generated with serial dilutions of human MMP-2 protein of known concentrations.

Statistical analysis

All data were expressed as ($\overline{x}$ ± s) and were analyzed by SPSS 13.0 software (SPSS Inc, Chicago, USA). Differences between the means of every pair of groups were detected using Student's *t*-test. $P < 0.05$ were considered statistically significant. All experiments assays were performed with three independent experiments.

Results

HGF can promote the invasiveness of SKOV-3 cells in vitro

After HGF treatment for 24 h, compared with the HGF stimulation and control groups, the number of migrating cells in the stimulation group increased. There was no significant difference in the number of migrating cells between the 40 and 80 ng/ml concentration groups, indicating that with an increasing concentration of HGF, the invasiveness of SKOV-3 cells gradually increased in a concentration-dependent manner within a certain range (**Figure 1**).

HGF can up-regulate the expression of PARP-1 in SKOV-3 cells

After HGF treatment for 24 h, compared with the 20, 40, 60, or 80 ng/ml HGF stimulation and control groups, *PARP-1* mRNA levels increased; the relative expression of *PARP-1* mRNA in each group was 0.34 ± 0.08, 0.52 ± 0.06, 0.78 ± 0.11, and 0.82 ± 0.09, respectively (**Figure 2A**). There was no difference in *PARP-1* mRNA levels between the 60 and 80 ng/ml HGF concentration groups, indicating that when the concentration of HGF increased, *PARP-1* mRNA expression levels in SKOV-3 cells gradually increased in a concentration-depen-

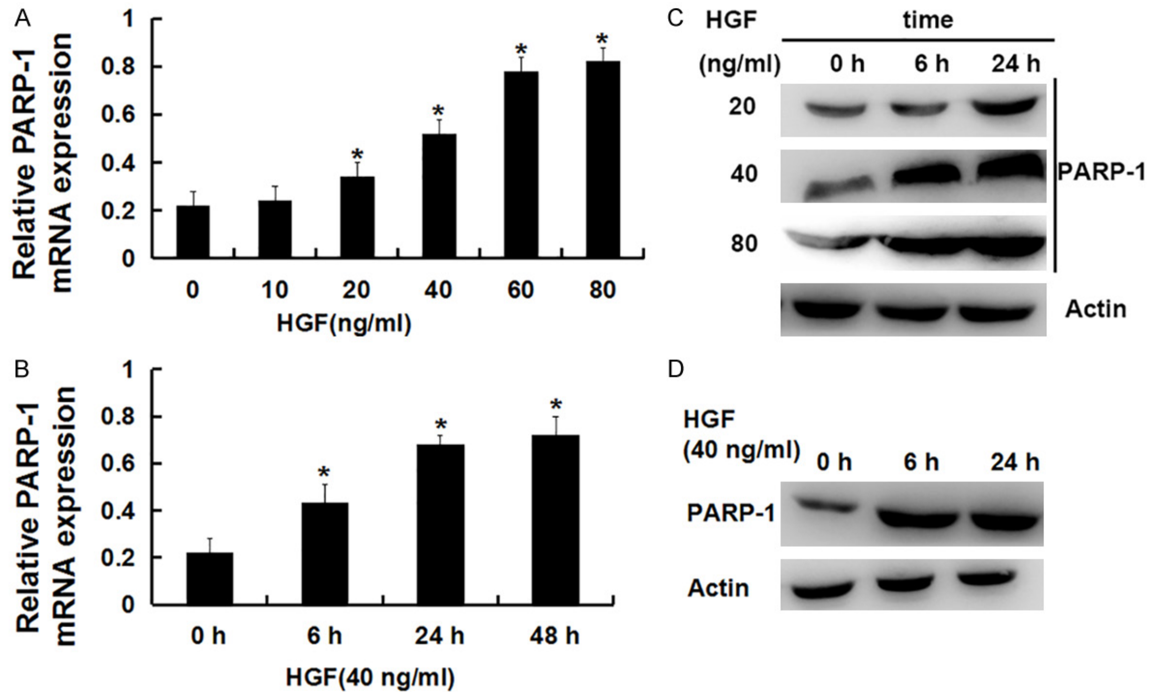

Figure 2. Expression of PARP-1 after HGF treatment in SKOV-3 cells. A. RT-PCR analysis of PARP-1 mRNA expression in SKOV-3 cells, which were exposed to HGF at the indicated concentrations. B. Time-dependent upregulation of PARP-1 in SKOV-3 cells at indicated time points. C, D. Western blot analysis of protein expression of PARP-1; $^{*}P < 0.05$ vs. control.

dent manner within a certain range. After HGF treatment for 6 h, compared with the normal control group, levels of *PARP-1* mRNA expression significantly increased. Levels of *PARP-1* mRNA expression gradually increased with the half-life of HGF, and this increase was statistically significant, suggesting that the regulation of *PARP-1* mRNA expression levels by HGF was time-dependent (**Figure 2B**). After 20, 40, or 60 ng/ml HGF treatment of SKOV-3 cells, protein was extracted after treatment for 0, 6, 24, 48 h, and western blotting was used to measure the expression of PARP-1 protein. We found that changes in PARP-1 protein levels were consistent with the changes that we observed in mRNA levels (**Figure 2C**). Expression levels of PARP-1 protein expression gradually increased with the half-life of HGF (**Figure 2D**), indicating that HGF can upregulate the expression of PARP-1 in SKOV-3 cells at the mRNA and protein levels.

RNA interference-mediated silencing of PARP-1 in SKOV-3 cells

Because GFP was encoded by the lentiviral vectors, green fluorescence could be observed in successfully transfected cells using an inverted fluorescence microscope; fluorescent expression was visible after 12 h and the intensity was the highest at 72 h. The transfection efficiencies of the NC-siRNA and PARP-siRNA groups were 88% and 86%, respectively. Compared with the normal control group, PARP-1 mRNA expression in the PARP-siRNA group was significantly reduced ($P < 0.05$), while compared with the control and NC-siRNA groups, there was no difference in PARP-1 mRNA expression levels ($P > 0.05$; **Figure 3**).

Silencing the expression of PARP-1 can partially inhibit HGF-induced PARP-1 expression

The expression levels of *PARP-1* in the control, NC-siRNA, and PARP-siRNA groups were 1.09 ± 0.04, 1.06 ± 0.10, 0.47 ± 0.06, respectively; levels of PARP-1 protein expression were significantly lower than those of the control and NC-siRNA groups after transfection; after 40 ng/ml HGF treatment for 24 h, the expression levels of PARP-1 in the HGF, HGF+NC-siRNA, and HGF+PARP-siRNA groups were 1.38 ± 0.04, 1.40 ± 0.10, and 0.55 ± 0.02, respectively, and levels of PARP-1 protein expression

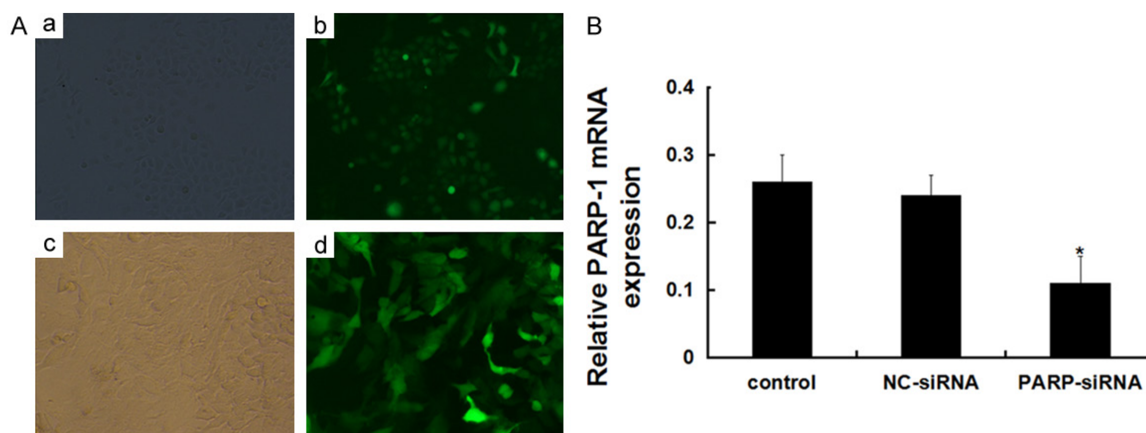

Figure 3. RNA interference-mediated silencing of PARP-1 in SKOV-3 cells. A. With the Green Fluorescent Protein reporter gene, fluorescence microscopy confirmed the transfection efficiencies of the NC-siRNA (a, b) and PARP-siRNA (c, d) (200×). B. RT-PCR analysis of PARP-1 mRNA expression in the NC-siRNA and PARP-siRNA groups; *$P < 0.05$ vs. control.

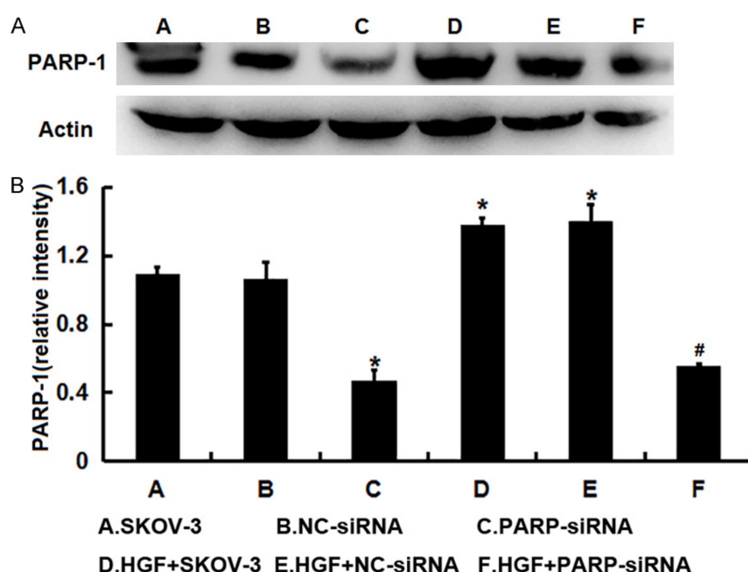

Figure 4. Expression of PARP-1 after HGF treatment in the NC-siRNA and PARP-siRNA groups. A. Extracted proteins were analyzed by western blot in different experimental groups. The staining intensity of actin indicated comparable protein loadings in each sample. B. Densitometry shows means ± SD, *$P < 0.05$ vs. A and B groups; #$P < 0.05$ vs. C group.

were significantly enhanced (**Figure 4**) compared with those of the HGF+NC-siRNA and HGF+PARP-siRNA groups (P < 0.05), indicating that HGF can upregulate the expression of PARP-1 in SKOV-3 cells at the protein level.

Silencing the expression of PARP-1 can partially inhibit the HGF-induced increased invasion ability of SKOV-3 cells

The transwell invasion assay results indicated that the mean number of migrating cells in the NC-siRNA and PARP-siRNA groups were 55.80 ± 2.59 and 38.20 ± 1.92, respectively. The mean number of migrating cells in the PARP-siRNA group was significantly reduced compared with that of the NC-siRNA group (P < 0.05). After HGF treatment, the number of migrating cells in each group significantly increased to 88.60 ± 2.07 and 72.00 ± 3.16 in the GF+NC-siRNA and HGF+PARP-siRNA groups, respectively (P < 0.05; **Figure 5**).

Silencing PARP-1 can partially inhibit the HGF-induced increased levels of MMP-2

The concentration of MMP-2 in cell supernatants was measured by ELISA, and the MMP-2 concentrations in the supernatants of the PARP-siRNA and NC-siRNA groups were 191.00 ± 7.75 and 296.50 ± 7.05 pg/ml, respectively. The amounts in the PARP-siRNA group were significantly lower than those in the NC-siRNA group, suggesting that inhibiting PARP-1 can significantly reduce the expression levels of MMP-2. There was no significant difference in MMP-2 levels between the NC-siRNA and non-transfection groups. After HGF treatment, the cell-induced MMP-2 secretion levels in the HGF+NC-siRNA and HGF+PARP-

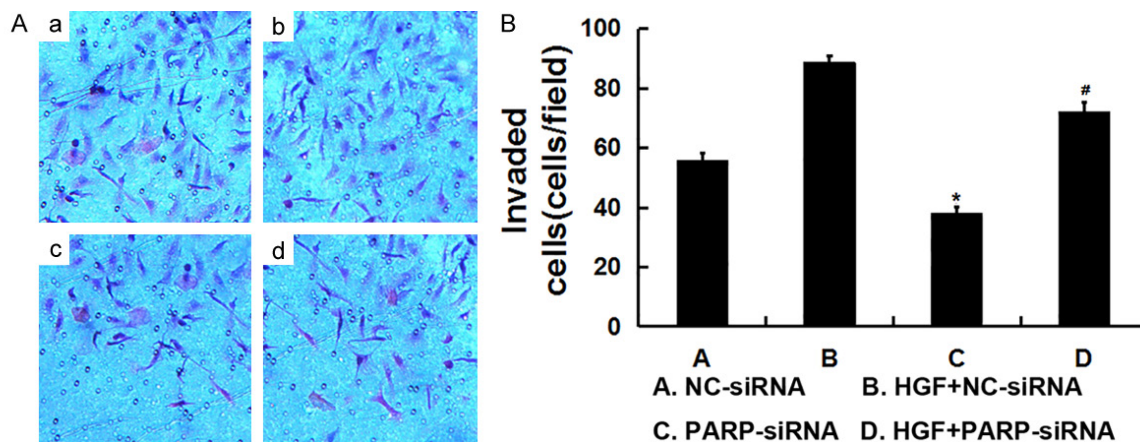

Figure 5. Effects of PARP-I on HGF-regulated invasiveness in SKOV-3 cells. A. Micrographs depicting a random view field on the lower side of the transwell chambers in different experimental groups (100×). B. Graphs show the ability of cell invasion in NC-siRNA and PARP-siRNA cells exposed to HGF for 24 h; **P* < 0.05 vs. A group; #*P* < 0.05 vs. B group.

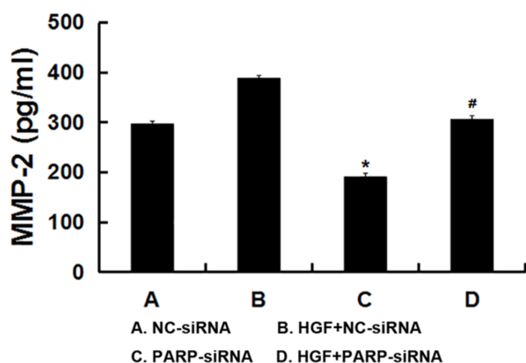

Figure 6. PARP-1 modulates the secretion of MMP-2 in HGF-stimulated SKOV-3 cells. Cell supernatants were assayed for MMP-2 content by ELISA. Data are expressed as mean concentrations (pg/mL) of protein in the supernatant ± SD; **P* < 0.05 vs. A group; #*P* < 0.05 vs. B group.

siRNA groups were 387.80 ± 14.55 and 306.70 ± 12.25 pg/ml, respectively (**Figure 6**), and the difference between these levels was significant (P < 0.05). Thus, in the HGF+PARP-siRNA group, the amounts of HGF-induced cell invasion and MMP-2 levels were both significantly reduced, indicating that silencing PARP-1 can partially inhibit the HGF-induced enhancement of SKOV3 cell invasion capacity. The mechanism involved could be related to MMP-2.

Discussion

Ovarian cancer is one of the three most malignant tumors of the female reproductive system, in which epithelial ovarian carcinoma accounts for 85-90% of primary malignant ovarian tumors. Tumor invasion and metastasis are important factors that can affect the prognosis of patients with ovarian cancer; therefore, studies of the mechanism of invasion and metastasis of tumor cells are important for improving the prognosis of patients.

Previous studies of PARP-1 have focused on DNA repair, gene regulation, and the regulation of cell proliferation and apoptosis. Recently, the role of PARP-1 in tumorigenesis and malignant progression has increasingly attracted the attention of researchers [7]. The expression and activities of PARP-1 in breast cancer, prostate cancer, pancreatic cancer, and other tumors were found to be enhanced. PARP-1 also plays an important role in the occurrence and development of tumors [8]. Our previous study found that PARP-1 gene silencing or the application of the PARP-1 inhibitors 3-AB and PJ34 could inhibit ovarian cancer cell proliferation *in vitro* [9].

Studies have shown that the influence of HGF on tumor cells is multifaceted, as it involves all aspects of biological behaviors, such as proliferation and invasion [10-12], resulting in the malignant transformation and metastasis of tumor cells. HGF was robustly expressed in liver cancer, breast cancer, colon cancer, lung cancer, and other tissues [13, 14]. This expression pattern indicated a strong role in promoting cell division and tissue morphogenesis, and in inducing epithelial cell migration, invasion, and

angiogenesis [15]. Therefore, the role of HGF in the malignant progression of cancer has become a focus in the field of oncology [16]. However, the specific mechanism of action of HGF is not yet fully understood. To further clarify the relationship between HGF and PARP-1 and its role in the malignant progression of ovarian cancer, we first explored the regulation of HGF in PARP-1 expression in ovarian cancer cells and the effects of PARP-1 in HGF-induced cell invasion behavior.

Tumor invasion and metastasis are closely related to extracellular matrix degradation and changes in cell migration invasion ability. The MMPs are an important group of extracellular matrix-degrading enzymes that play critical roles in the invasion and metastasis of tumor cells by degrading different components of the extracellular matrix [17]. Studies have shown that MMP-2 is overexpressed in many malignant tumors, and its expression levels are significantly associated with both the widespread metastasis of advanced ovarian cancer and a poor prognosis [18, 19].

Our study found that HGF could enhance the *in vitro* invasion ability of SKOV-3 cells and increase MMP-2 secretion. HGF-induced cell invasion and MMP-2 secretion were significantly reduced after silencing PARP-1, but were not completely inhibited. This finding suggested that one mechanism of action of HGF might be to promote cell invasion by regulating the expression of PARP-1, thereby mediating an increase in MMP-2 levels, consistent with previous findings. Mariani et al. [20] confirmed that HGF could stimulate the invasion and metastasis of ovarian cancer cells, thereby promoting peritoneal implantation and ovarian cancer metastasis. Matsui et al. [21] found that inhibiting the HGF/c-Met signal transduction pathway could reduce the metastasis of colorectal cancer. Ramanujum et al. [22] reported that HGF significantly increased the proliferation, migration, and invasion capacity of lung cancer cells, which was related to an increase in the levels of MMPs secreted by tumors. Kim et al. [23] reported that PARP-1 could regulate the secretion of MMPs and promote the lymph node metastasis of gastric cancer cells, which was consistent with our findings.

We transfected PARP-siRNA into ovarian cancer cells, and showed for the first time that silencing PARP-1 expression could partially inhibit the malignant biological characteristics of SKOV-3 cells induced by HGF. Therefore, we inferred that the mechanism of HGF inducing the invasion behavior of ovarian cancer cells could be related to PARP-1-mediated upregulation of MMP-2. This study sought to further characterize the interaction between HGF and PARP-1 in the malignant progression of ovarian cancer and its molecular mechanisms, with the aim of providing a theoretical basis and experimental evidence for a control strategy for ovarian cancer based on the downstream pathways as targets.

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Disclosure of conflict of interest

None.

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