Abstract. Tumor metastasis is the leading cause of mortality in patients with advanced ovarian cancer. Myelin protein zero like 1 (MPZL1) is a transmembrane glycoprotein that promotes migration of hepatocellular carcinoma cells and is involved in extracellular matrix-induced signal transduction. However, the functional role of MPZL1 in ovarian cancer has not been well elucidated. The present study conducted western blotting, phase-contrast imaging and immunohistochemistry to reveal the functions of MPZL1 in ovarian cancer. The present study demonstrated that the expression levels of MPZL1 were associated with malignant features of ovarian cancer. Furthermore, overexpression of MPZL1 significantly promoted cell proliferation, migration and invasion of ovarian cancer cells. Conversely, MPZL1 depletion by short hairpin RNA inhibited migration and invasion of ovarian cancer cells. In addition, this study demonstrated that phosphorylation of Src kinase was increased upon MPZL1 overexpression. Additionally, phosphorylation and activation of pro-metastatic proteins p130 and cortactin were induced by phosphorylated Src kinase. Collectively, these findings indicated that MPZL1 may be a novel pro-metastatic gene, which promotes tumor cell proliferation and migration through Src-mediated phosphorylation of p130 and cortactin in ovarian cancer.

Introduction

Ovarian cancer has the highest mortality rate among gynecological malignancies worldwide (1,2). Tumor metastasis is a complex biological process involving cell signaling, regulation of cell proliferation, motility and invasion; in addition, metastasis is the primary cause of death in patients with advanced ovarian cancer and is closely associated with unfavorable outcomes and poor prognosis (3-7). Recently, the rat sarcoma-mitogen-activated protein kinase, phosphoinositide 3-kinase-protein kinase B and janus kinase-signal transducer and activator of transcription signaling pathways have been reported to be associated with tumor metastasis and invasion in ovarian cancer (8). Furthermore, the tumor microenvironment, which includes stromal cells, extracellular matrix components and exosomes, can establish a communication circuit that enhances cancer cell invasion and metastasis via reciprocal signaling (9). However, the molecular mechanisms underlying ovarian cancer metastasis are currently not well elucidated (10).

Myelin protein zero like 1 (MPZL1), also known as protein zero-related, is a hyperphosphorylated transmembrane glycoprotein involved in extracellular matrix-induced signal transduction (11-15). Previous studies have demonstrated that MPZL1 promotes hepatocellular carcinoma cell migration through the Src tyrosine kinase, and may be involved in adhesion-dependent signaling (11,14,16). Furthermore, MPZL1 forms a complex with the growth factor receptor-bound protein 2 adaptor and tyrosine-protein phosphatase non-receptor type 11 phosphatase, and is involved in cell adhesion in human epidermal growth factor receptor 2-positive breast cancer cells (12). Additionally, as a major receptor of concanavalin A, MPZL1 has an important role in cell signaling via c-Src (13). However, the functional role and clinical implications of MPZL1 in ovarian cancer are largely unknown.

The present study demonstrated that amplification of MPZL1 was associated with malignant features of ovarian cancer, and promoted tumor cell proliferation, migration and invasion. Furthermore, overexpression of MPZL1 significantly promoted cell growth and metastasis of ovarian cancer. Conversely, knockdown of MPZL1 via short hairpin RNA (shRNA) attenuated proliferation and migration of ovarian cancer cells. This study also demonstrated that MPZL1 overexpression-induced activation of Src kinase mediated the phosphorylation and activation of cortactin and p130 in ovarian cancer. Taken together, these findings suggested that MPZL1 may be considered a novel pro-metastatic gene that promotes tumor cell proliferation and migration via activation of Src kinase in ovarian cancer.
Materials and methods

Collection of ovarian cancer specimens. The present study was approved in January 2017 by the Ethics Committee of the Tongren Hospital, Shanghai Jiao Tong University School of Medicine. The collection of ovarian cancer specimens was performed in conformity to ethical standards. All participants provided written informed consent. The tissues were obtained during surgery and were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 24 h at room temperature and embedded in paraffin. Specimens were obtained from 78 patients (age, 29-77 years, including 16 benign, 16 borderline and 46 malignant patients) with epithelial ovarian cancer who had not previously undergone treatment; the benign group consisted of patients with a benign ovarian tumor. No distant metastasis was detected in the selected patients prior to surgery. Detailed pathological data, including histological type, tumor size, tumor stage and lymph node metastasis, were obtained and summarized. The tumor stage was defined using the 8th edition of the Union for International Cancer Control-tumor node metastasis system (17).

Reagents and cell lines. The human ovarian cancer cell lines 293T, HO8910, SKOV3, HEY and TOV-21G were purchased from American Type Culture Collection and were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.).

Virus production and infection. 293T cells (60% confluent) in a 10 cm dish were co-transfected with 5 µg lentiviral constructs (pLenti 7.3; Invitrogen; Thermo Fisher Scientific, Inc.), 5 µg plasmid Δ8.9 (Invitrogen; Thermo Fisher Scientific, Inc.) and 3 µg plasmid vesicular stomatitis virus G (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.).

Western blotting. Cells were lyzed in RIPA buffer [Tris (pH 7.4), 50 mM; NaCl, 150 mM; 1% NP-40; 0.1% SDS; EDTA, 2 µM] containing protease inhibitors (Roche Molecular Diagnostics) and phosphatase inhibitors (Roche Molecular Diagnostics), and the protein concentration was determined using the bicinchoninic acid assay. The cell lysates (20 µg total protein) were subjected to 8-10% SDS-PAGE and immunoblotting. Subsequently, proteins were transferred to nitrocellulose membranes, which were blocked for 1 h with 5% nonfat milk at room temperature, and were then incubated with primary antibodies (1:1,000) at 4°C overnight. The membranes were then incubated for 1 h with horseradish peroxidase-linked anti-rabbit IgG antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc) at room temperature. Blots were visualized with ECL western blotting reagents (Thermo Fisher Scientific, Inc.) using ChemiDoc XRS+ (Bio-Rad Laboratories, Inc.). Semi-quantification was conducted using ImageLab 2.0 software Bio-Rad Laboratories, Inc.). Antibodies against the following proteins were used: p130 (cat. no. 13846), phosphorylated (p)-p130 (cat. no. 4015), cortactin (cat. no. 3502), p-cortactin (cat. no. 4569), Src (cat. no. 2108), p-Src (cat. no. 12432), MPZL1 (cat. no. 9893) and GAPDH (cat. no. 5174) (all Cell Signaling Technology, Inc). Notably, since MPZL1 has three alternatively spliced isoforms, at least three bands can be seen in MPZL1 blots, and all bands were measured when semi-quantifying the blots.

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). Cells (5,000 cells/well) were seeded in triplicate in 96-well plates. After 24, 48, 72, 96 or 120 h at 37°C, CCK-8 reagent (1/20, volume/volume) was added to the cells for 2 h at 37°C. The absorbance of each well was measured at 450 nm, according to the manufacturer's protocol.

Cell migration and invasion assays. Transwell chambers with 8 µm pore membranes (Corning, Inc.) were placed in 24-well culture plates and were incubated with serum-free RPMI-1640 medium at 37°C for 1 h. A 200 µl suspension of 0.5-1x10^5 cells was seeded into the upper compartment of the Transwell chambers in FBS-free medium, whereas 600-800 µl RPMI-1640 medium supplemented with 10% FBS was added into the bottom wells; cells were incubated at 37°C for 12-24 h. The migrating cells that were attached to the lower membranes of the Transwell chambers were stained with crystal violet (0.1%) at room temperature for 2 h and images were captured at x200 magnification under a light microscope. Images of three random fields from three replicate wells were obtained, and the migrated cells were counted. The cell invasion assay was the same as the migration assay; however, the chambers were coated with Matrigel.

Colony formation assay. Cells were seeded in triplicate in 6-well plates. After 12 days, the cells were washed with PBS, fixed with methanol for 1 h and stained with 0.1% crystal violet for 1 h at room temperature. Subsequently, images of the colonies were captured and counted.

Immunohistochemistry. Ovarian cancer specimens were collected from the 78 patients during surgery at Tongren.
Hospital, Shanghai Jiao Tong University. The tissue specimens were fixed in 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 24 h at room temperature and embedded in paraffin, before being cut into 5 µm sections. The tissue sections were deparaffinized, treated with 3% H₂O₂ for 10 min at room temperature, autoclaved in 10 mM citric sodium (pH 6.0) for 30 min to unmask antigens and rinsed in PBS. Subsequently, sections were incubated with primary antibodies against MPZL1 (1:200; cat. no. GTX46451; GeneTex, Inc.) at 4°C overnight, followed by incubation with a biotinylated secondary antibody (1:1,000; cat. no. ab6844; Abcam) for 1 h at room temperature. Signal amplification and detection was performed using the DAB system (Dako) according to the manufacturer’s instructions.

Immunohistochemistry scoring. All cases were analyzed independently with the help of two expert pathologists and were assigned a score according to the following criteria: Strongly positive, 3+; positive, 2+; weakly positive, 1+; and negative, 0.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). Genomic analysis of MPZL1 in ovarian cancer (489 high-grade serous ovarian cancer specimens were surgically resected prior to systemic treatment; all patients received a platinum agent and 94% received a taxane) was performed with The Cancer Genome Atlas (TCGA) copy number portal (www.broadinstitute.org/tcga). In all experiments, comparisons between two groups were conducted using two-sided Student’s t-test, and one-way analysis of variance followed by Tukey’s multiple comparisons test was used to test for differences among more groups. Fisher’s exact test was used to determine differences between stages, as presented in Table I. P<0.05 was considered to indicate a statistically significant difference.

Results
Overexpression of MPZL1 is associated with malignant features of ovarian cancer. Copy number analysis of TCGA ovarian serous adenocarcinoma samples in the cBioportal database revealed that MPZL1, located at chromosome 1q24 (magnified section in Fig. 1A), was genomically amplified in a considerable proportion of cases (Fig. 1A). Notably, the expression levels of MPZL1 were associated with its amplification status (Fig. 1B), thus indicating that the high expression of MPZL1 in tumor tissues may be regulated by copy number amplification of the MPZL1 gene. To further determine the association of MPZL1 expression with the malignant features of ovarian cancer, MPZL1 protein expression was assessed by immunohistochemistry among benign, borderline and malignant epithelial ovarian cancer patient tissues (Fig. 1C; Table I). The results revealed that MPZL1 expression was almost undetectable in patients with benign ovarian cancer, whereas it was expressed at significantly higher levels in malignant cancer patient tissues compared with in benign/borderline cancer patient tissues (Fig. 1D). In addition, the expression profile of the MPZL1 protein was determined using The Human Protein Atlas (18). MPZL1 was ubiquitously expressed in different human tissues as well as various cancer types. In normal ovarian tissues, MPZL1 was lowly expressed, whereas in some ovarian cancer tissues, MPZL1 was overexpressed (data not shown). Taken together, these data indicated that MPZL1 was overexpressed in a subset of patients with ovarian cancer, suggesting that MPZL1 may serve a pivotal role in ovarian cancer.

Overexpression of MPZL1 promotes ovarian cancer cell proliferation and migration. To study the biological function of MPZL1, the protein expression levels of MPZL1

| Variable         | Cases (n) | Low level | High level | P-value |
|------------------|-----------|-----------|------------|---------|
| Age (years)      |           | -         | +          |         |
| <55              | 28        | 1         | 8          | 9       | 10     | 0.69 |
| ≥55              | 18        | 0         | 4          | 4       | 10     |       |
| Clinical stage   |           |           |            |         |         |       |
| I-II             | 9         | 1         | 5          | 2       | 1      | 0.01*|
| III              | 25        | 0         | 5          | 8       | 12     | 0.02*|
| IV               | 12        | 0         | 2          | 3       | 7      |       |
| CA125 (U/ml)     |           |           |            |         |         |       |
| ≤200             | 33        | 1         | 12         | 10      | 10     |       |
| >200             | 13        | 0         | 0          | 3       | 10     | 0.01 |

*Stage III vs. stage I-II; †stage IV vs. stage III. Data were analyzed by Fisher’s exact test. CA125 cancer antigen 125; MPZL1, myelin protein zero like 1.
were detected in four ovarian cancer cell lines (Fig. 2A). Subsequently, the *MPZL1* gene was overexpressed via lentiviral infection in SKOV3 and TOV-21G cells (Fig. 2B); these two cell lines were selected as they exhibited relatively lower endogenous MPZL1 expression. Subsequently, the effects of MPZL1 overexpression on the proliferation of these ovarian cancer cells were determined using the CCK-8 assay. Notably, the results demonstrated that exogenous overexpression of the *MPZL1* gene promoted ovarian cancer cell proliferation (Fig. 2C). Similarly, the results of the colony formation assay demonstrated that the number of colonies was significantly increased in *MPZL1*-overexpressed SKOV3 and TOV-21G cells (Fig. 2D). Furthermore, the effects of MPZL1 overexpression were examined on the migratory and invasive abilities of ovarian cancer cells by Transwell assays; ectopic expression of MPZL1 significantly enhanced the *in vitro* migration and invasion of ovarian cancer cells (Fig. 2E). These results indicated that MPZL1 may serve an important role in promoting cell migration and metastasis of ovarian cancer.

**Targeted downregulation of MPZL1 attenuates ovarian cancer cell proliferation and migration.** To further verify the role of MPZL1 in ovarian cancer cell migration, HO8910 and HEY cells were selected as cellular models for loss-of-function studies. Firstly, the MPZL1 gene was stably knocked down in HO8910 and HEY cells using a lentiviral shRNA specifically targeting MPZL1 (Fig. 3A). Consequently, cell proliferation was significantly inhibited under normal growth conditions (Fig. 3B). Consistently, the colony formation assay revealed that the number of colonies was evidently decreased following knockdown of MPZL1 in HO8910 and HEY cells (Fig. 3C). Furthermore, Transwell assays demonstrated that the *in vitro* migration and invasion of ovarian cancer cells...
were inhibited by MPZL1 depletion (Fig. 3D). These findings indicated that MPZL1 knockdown attenuated proliferation, migration and invasion of ovarian cancer cells.

**MPZL1 regulates phosphorylation levels of numerous pro-metastatic proteins.** Recently, many studies have reported that pro-metastatic proteins, including p130, Src and cortactin, are important for cell migration and tumor metastasis (19-21). Furthermore, p130 and cortactin were originally identified as substrate proteins of the Src family kinases, and a previous report demonstrated that the MPZL1/Src/cortactin signaling cascade functions in the process of hepatocellular carcinoma cell migration (22).

However, to the best of our knowledge, the potential role of MPZL1 in the phosphorylation of pro-metastatic proteins has not been determined in ovarian cancer. To investigate whether the MPZL1/Src/cortactin signaling cascade exists in ovarian cancer, the expression levels of pro-metastatic proteins were detected by western blotting. The results indicated that stable overexpression of MPZL1 led to increased phosphorylation of p130, Src and cortactin in SKOV3 cells (Fig. 4A). Furthermore, targeted knockdown of the MPZL1 gene by shRNA reduced phosphorylation of these three proteins in HO8910 cells (Fig. 4B). These findings indicated that MPZL1 overexpression reprogrammed the pro-metastatic signaling network in ovarian cancer.
Transient transfection of Src siRNA suppresses migration and invasion in SKOV3-MPZL1 cells. According to previous studies, Src tyrosine kinases serve critical roles in several cellular signal transduction pathways that regulate cell proliferation, adhesion, migration and invasion (22-25). Additionally, as substrate proteins of Src kinase, p-p130 and p-cortactin participate in cell migration (26). Furthermore, amplification of MPZL1 promotes tumor cell migration via Src-mediated cortactin phosphorylation in hepatocellular carcinoma cells (27). To ascertain whether Src kinase activity is essential for MPZL1-induced phosphorylation of p130 and cortactin in ovarian cancer cells, MPZL1 was overexpressed in SKOV3 cells, and the Src gene was then knocked down by siRNA. Notably, stable overexpression of MPZL1 significantly increased phosphorylation of p130, cortactin and Src in SKOV3-MPZL1 cells, and enhanced the migratory and invasive abilities of SKOV3-MPZL1 cells (Fig. 5A-C). Conversely, targeted knockdown of Src via siRNA resulted in reduced phosphorylation of p130 and cortactin, and suppressed the migration and invasion of SKOV3-MPZL1 cells (Fig. 5A-C). Taken together, these findings indicated that Src kinase activity may be essential for MPZL1-mediated migration and invasion of ovarian cancer cells.

Discussion

In the present study, amplified MPZL1 was associated with the malignant features of ovarian cancer. Subsequently,
overexpression and knockdown of MPZL1 indicated that MPZL1 served a prominent role in the promotion of ovarian cancer cell proliferation, migration and invasion. In addition, amplification of MPZL1 promoted tumor cell migration through Src-mediated phosphorylation of p130 and cortactin. Therefore, these data suggested that MPZL1 may operate as a novel oncoprotein in ovarian cancer.

Previous studies have reported that the MPZL1 protein is involved in cell signaling, proliferation, differentiation and transformation (15,28). Furthermore, amplification of MPZL1 promotes tumor cell migration through Src-mediated phosphorylation of cortactin in hepatocellular carcinoma (27,29). However, the biological functions and clinical implications of MPZL1 in other types of human cancer remain unclear. This study identified a positive association between the protein expression levels of MPZL1 and cell proliferation, migration and invasion of ovarian cancer cells.

Src family protein tyrosine kinases participate in numerous signaling pathways that control cellular responses, including proliferation, survival, adhesion and migration in normal and cancer cells (30-33). As substrate proteins of Src kinase, p130 and cortactin become tyrosine-phosphorylated during integrin-mediated cell adhesion to the extracellular matrix, cell attachment and cell invasion (24,34-36). Furthermore, it has been demonstrated that tyrosine-phosphorylated cortactin via Src kinase, which is activated upon MPZL1 overexpression, increases the migratory potential of hepatocellular carcinoma cells (14,27). In this study, it was demonstrated that overexpression of MPZL1 resulted in increased phosphorylation of Src, p130 and cortactin. Additionally, the migration and proliferation of ovarian cancer cells were promoted. These findings indicated that MPZL1 may serve an important role in ovarian cancer cell metastasis. However, the physiological relevance of these data requires further mechanistic investigations and in vivo studies.

In conclusion, the present results suggested that amplification of MPZL1 in ovarian cancer may be associated with the malignant features of ovarian cancer. MPZL1 was involved in Src-mediated phosphorylation of p130 and cortactin, thereby promoting ovarian cancer cell proliferation, migration and invasion. Furthermore, these data identifies MPZL1 as a novel pro-metastatic gene in ovarian cancer. Together with a report implicating MPZL1 in other cancer types (27), the present study expands our understanding of human ovarian cancer metastasis and indicates potential therapeutic avenues for the treatment of ovarian cancer.

Figure 4. MPZL1 regulates the phosphorylation of numerous pro-metastatic proteins. (A) Overexpression of MPZL1 in SKOV3 cells increased the phosphorylation of p130, cortactin and Src. ***P<0.001 vs. vector. (B) Knockdown of MPZL1 by shRNA in HO8910 cells decreased the phosphorylation of p130, cortactin and Src. Phosphorylated proteins were normalized to both total proteins and GAPDH. Each group consisted of three biological replicates and representative images are shown. Data are presented as the mean ± SEM. ***P<0.001 vs. NC. MPZL1, myelin protein zero like 1; NC, negative control; p-, phosphorylated; sh/shRNA, short hairpin RNA.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

XW and DC designed and conceived the experiments. LC analyzed the data. XW, DC and LC drafted the manuscript and wrote the manuscript. XW supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the Tongren Hospital, Shanghai Jiao Tong University School of Medicine. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure 5. Transient transfection of Src siRNA decreases cell migration and invasion in SKOV3-MPZL1 cells. (A) Western blot analysis following transient transfection of Src siRNA in SKOV3-MPZL1 cells; control cells were transfected with NC siRNA. Phosphorylated proteins were normalized to both total proteins and GAPDH. (B) Transwell migration assays of SKOV3 cells (magnification, x200). (C) Transwell invasion assays of SKOV3 cells (magnification, x200). Each group consisted of three biological replicates. Data are presented as the mean ± SEM. **P<0.01, ***P<0.001 vs. MPZL1 or as indicated. MPZL1, myelin protein zero like 1; p-, phosphorylated; siRNA, small interfering RNA.
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