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Original Paper

Placental Growth Factor Promotes Metastases of Ovarian Cancer Through MiR-543-Regulated MMP7

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Key Words
Ovarian cancer (OC) • Placental growth factor (PLGF) • Matrix metalloproteinase -7 (MMP7) • MiR-543

Abstract
Background/Aims: Elucidation of the molecular mechanisms underlying ovarian cancer (OC) cell invasion and migration may provide important evidence for developing efficient therapy. Recently, Matrix metalloproteinase (MMP) has been shown to be regulated by vascular endothelial growth factor family members, especially placental growth factor (PLGF), as a potential mechanism underlying cancer invasion. Here, we studied the molecular relationship between PLGF and MMP7 in the OC. ***Methods:*** We examined the levels of PLGF and MMP7 in the resected OC specimens and compared to paired adjacent non-tumor ovarian tissue. We also examined the correlation between PLGF and MMP7. We modified PLGF levels in a human OC cell line, OVCAR3, and analyzed the effects on MMP7. Prediction of microRNA (miRNA) binding to 3'-UTR of MMP7 mRNA was performed by bioinformatics analyses and confirmed by a dual luciferase reporter assay using miR-543-modifed OC cells. The levels of miR-543 were examined in the OC specimens, and the correlation between miR-543 and PLGF or MMP7 was performed. ***Results:*** PLGF and MMP7 both significantly increased in the OC specimens, compared to paired adjacent non-tumor ovarian tissue. PLGF significantly increased MMP7 in the OC cells at protein level, but not at mRNA level. In OC cells, PLGF significantly decreased the levels of miR-543, which suppressed the translation of MMP7 mRNA via 3'-UTR binding. In OC specimen, miR-543 significantly decreased, compared to paired adjacent non-tumor ovarian tissue. An inverse correlation was detected between the levels of miR-543 and PLGF or MMP7 in the OC specimens. ***Conclusion:*** MiR-543 inhibits translation of MMP7 through binding to the 3'-UTR of MMP7 mRNA in OC. PLGF suppresses miR-543, which activates MMP7-mediated cancer invasion.

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Introduction

Ovarian cancer (OC) is a relatively rare cancer and accounts for only about 3% of all cancers among women. However, OC is generally more lethal, and causes more deaths than other cancers of the female reproductive system, resulting in a poor 5-year survival ratio as low as about half. This poor prognosis of OC largely rests on the highly aggressive nature of OC cells, in which early cancer invasion and metastases often occur [1, 2]. Hence, elucidation of the molecular mechanisms underlying OC cell invasion and migration may provide important evidence for developing efficient therapy [3-7].

The growth and metastases of ovarian cancer have been found to involve activation of cancer-associated proteinases from host stromal cells or inflammatory cells to enable them to break through collagenous protein barriers. Matrix metalloproteinase (MMP) is the most important proteinase in cancer invasion. In OC, activation of MMP2, MMP9, MMP3 and MMP7 has been reported and was associated with cancer-related cell invasion and migration [8-14]. Among the MMPs, MMP7 is relatively less investigated [15-18]. Moreover, the regulation of MMP7 in OC is ill-defined. Recently, MMPs have been shown to be regulated by vascular endothelial growth factor (VEGF) family members, e.g. VEGF-A, and placental growth factor (PLGF) [19-26]. VEGF family is the most potent triggers for vasculogenesis and angiogenesis during embryogenesis and in adults [27]. The VEGF family is composed of six secreted proteins: VEGF-a, VEGF-b, VEGF-c, VEGF-d, VEGF-e and PLGF [28-30]. Of note, PLGF plays an important role in the pathological angiogenesis, in a coordinated way with other VEGF family members [31-34]. Thus, recent findings of the regulation of MMPs by PLGF in cancer shed new light on the metastases-targeting therapy.

The recent studies have revealed an essential role of microRNAs (miRNAs) in the progression and metastases of various cancers. MiRNAs are non-coding RNAs that regulate many important biological events including cell growth, tissue differentiation, apoptosis and viral infection. Despite the short sequence length of miRNAs (about 22 nucleotides) and the relatively small number of microRNAs, about one third of all protein-coding genes are regulated by miRNAs, suggesting that miRNAs may act as master regulators for various biological pathways, including tumorigenesis [2, 35-42]. Among all miRNAs, miR-543 is also rarely studied. Until recently, the involvement of miR-543 in the carcinogenesis has been shown in endometrial cancer [43], in gastric cancer [44] and in breast cancer [45], but not in other cancers including OC.

Here, we showed that PLGF and MMP7 both significantly increased in the OC specimens, compared to paired adjacent non-tumor ovarian tissue. PLGF significantly increased MMP7 in the OC cells at protein level, but not at mRNA level. In OC cells, PLGF significantly decreased the levels of miR-543, which suppressed the translation of MMP7 mRNA via 3'-UTR binding. In OC specimen, miR-543 significantly decreased, compared to paired adjacent non-tumor ovarian tissue. An inverse correlation was detected between the levels of miR-543 and PLGF or MMP7 in the OC specimens.

Materials and Methods

Patient specimens

Resected OC specimens were collected from 25 OC patients (age: 27 to 54, median 43) in this study. OC specimens were compared with the paired adjacent non-tumor ovarian tissue (NOT) from the same patient. All specimens were histologically and clinically diagnosed at Shengjing Hospital of China Medical University from 2009 to 2014. Ovarian cancer vs. Not was determined by experienced pathologists. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Culture and transfection of human OC cell line

OVCAR3 is a human ovarian cancer cell line purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen,

St. Louis, MO, USA) supplemented with L-glutamine and 20% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO_2 at 37°C. The plasmids that express PLGF, or short hairpin interfering RNA for PLGF (shPLGF), or control scrambled sequence (scr), or miR-543, or antisense of miR-543 (as-miR-543), or control null sequence (null) were all purchased from Origene (Beijing, China). Transfection was performed with 2 μg plasmids using the Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). Cells were analyzed 48 hours after transfection.

Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from tissue specimen or from cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. Quantitative PCR was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to controls.

Western blot

For protein analysis, the protein was extracted from tissue specimens or cultured OC cells in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at 12000 × g at 4°C for 20min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are rabbit anti-MMP7 and anti-α-tubulin (Abcam, Cambridge, MA, USA). α-tubulin is used as a protein loading control. Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative from 5 individuals. NIH ImageJ software (Bethesda, MA, USA) was used for image acquisition and densitometric analysis of the gels.

ELISA

The concentration of PLGF and MMP7 was determined by human PLGF or MMP7 ELISA Kit (R&D System, Los Angeles, CA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition media was added to a well coated with primary antibody, and then immunosorbented by biotinylated primary antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Luciferase-reporter activity assay

Luciferase-reporters were successfully constructed using molecular cloning technology. Target sequence was inserted into pGL3-Basic vector (Promega, Madison, WI, USA) to obtain pGL3-MMP7-3'-UTR, which contains the miR-543 binding sequence (MMP7-3'-UTR sequence). OVCAR3-miR-543, or OVCAR3-null, or OVCAR3-as-miR-543 cells were seeded in 24-well plates for 24 hours, after which they were transfected with 1 μg of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Then luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to the manufacturer's instructions.

Statistics

All statistical analyses were carried out using the SPSS 19.0 statistical software package. All values are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction. Bivariate correlations were calculated by Spearman's rank correlation coefficients.

Results

OC expresses significantly higher levels of PLGF and MMP7, which are correlated

We examined the PLGF and MMP7 levels in the resected OC specimens from 25 patients, and compared to the paired adjacent non-tumor ovarian tissue (NOT). We detected significantly higher levels of PLGF by ELISA (Fig. 1A) and significantly higher levels of MMP7 by Western blot (Fig. 1B) in the OC specimens. Moreover, a strong correlation was detected between PLGF and MMP7 levels in OC specimens (Fig. 1C, R= 0.79; $p < 0.0001$), suggesting a causal relationship.

PLGF increases MMP7 at protein but not mRNA level

Since previous reports have shown a role of PLGF in regulation of MMPs, we were thus prompted to evaluate whether PLGF also regulate MMP7 levels in OC. Thus, we either overexpressed PLGF, or inhibited PLGF in a human OC cell line, OVCAR3, resulting in OVCAR3-PLGF or OVCAR3-shPLGF. OVCAR3 cells were also transfected with a plasmid carrying a scrambled sequence as a control (OVCAR3-scr). The modification of PLGF levels in OVCAR3 cells was confirmed by RT-qPCR (Fig. 2A). Then, we examined the levels of MMP7 in these PLGF-modified cells. We found that although the MMP7 transcripts did not change by PLGF modification (Fig. 2B), the protein levels of MMP7 in PLGF-overexpressing OVCAR3 cells was significantly increased, while the protein levels of MMP7 in PLGF-depleted OVCAR3 cells was significantly decreased, by Western blot (Fig. 2C), and by ELISA on secreted protein (Fig. 2D). These data suggest that PLGF may regulate MMP7 post-transcriptionally.

PLGF suppresses miR-543, which targets 3'-UTR of MMP7 mRNA in OC cells

Thus, we performed bioinformatics analyses of MMP7-target miRNAs, and specially found that miR-543 bound to 3'-UTR of MMP7 mRNA at 221^{th}-227^{th} base site (Fig. 3A). Moreover, the miR-543 levels were significantly decreased in PLGF-overexpressing OVCAR3 cells, while the miR-543 levels were significantly increased in PLGF-depleted OVCAR3 cells (Fig. 3B). To further prove that the binding of miR-543 to the 3'-UTR of MMP7 mRNA is functional, we modified miR-543 levels in OVCAR3 cells, by transfecting the cells with either miR-543 or antisense for miR-543 (as-miR-543). OVCAR3 cells were also transfected with null sequence as a control (null). Modulation of miR-543 levels in OVCAR3 cells was confirmed by RT-qPCR (Fig. 3C). Then, OVCAR3-miR-543, OVCAR3-null and OVCAR3-as-miR-543 cells were transfected with 1μg of MMP7-3'-UTR Luciferase-reporter plasmid. We

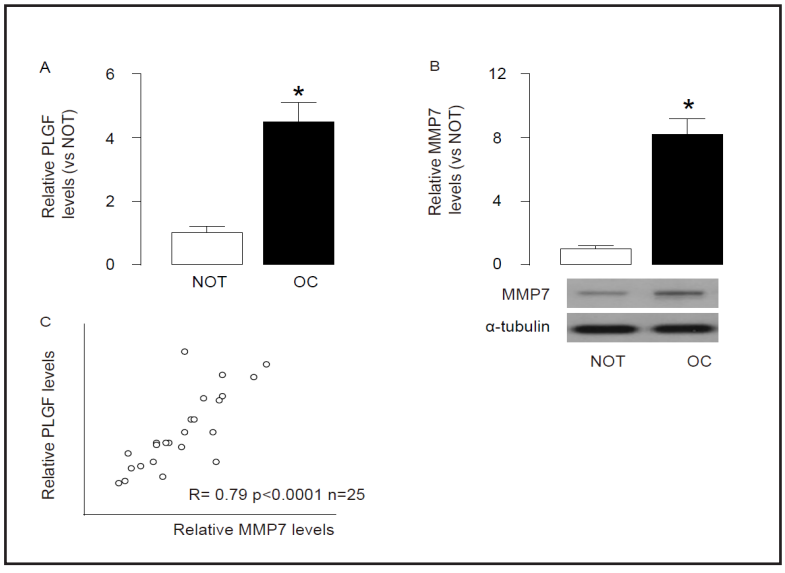

Fig. 1. OC expresses significantly higher levels of PLGF and MMP7, which are correlated. (A-B) We examined the PLGF levels by ELISA (A) and MMP7 levels by Western blot (B) in the resected OC specimens from 25 patients, and compared to the paired adjacent non-tumor ovarian tissue (NOT). (C) A strong correlation was detected between PLGF and MMP7 levels in OC specimens (R= 0.79; $p < 0.0001$). *$p < 0.05$. N = 25.

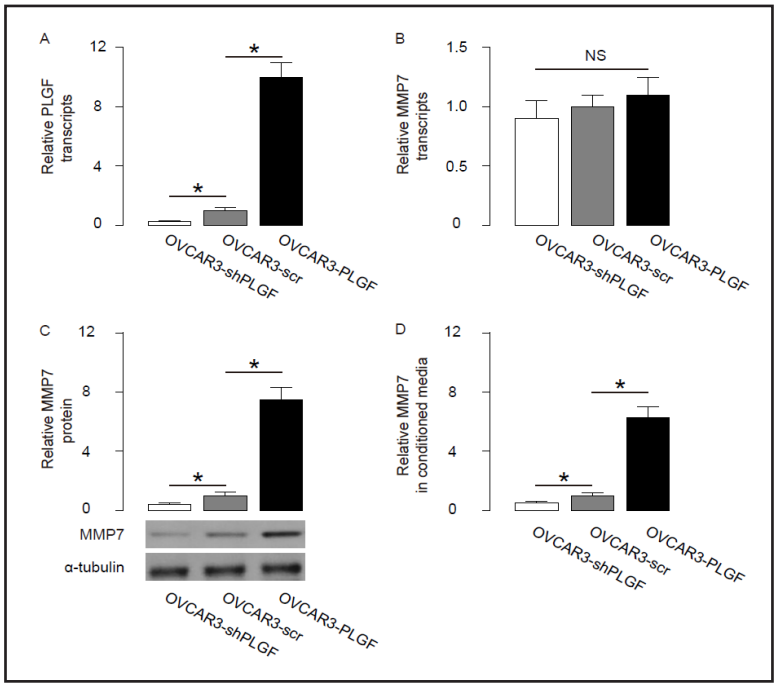

Fig. 2. PLGF increases MMP7 at protein but not mRNA level. We either overexpressed PLGF, or inhibited PLGF in a human OC cell line, OVCAR3, resulting in OVCAR3-PLGF or OVCAR3-shPLGF. OVCAR3 cells were also transfected with a plasmid carrying a scrambled sequence as a control (OVCAR3-scr). (A) The modification of PLGF levels in OVCAR3 cells was confirmed by RT-qPCR. (B-D) The levels of MMP7 in PLGF-modified cells, by RT-qPCR (B), by Western blot (C), and by ELISA on secreted protein (D). *p <0.05. NS: non-significant. N = 5.

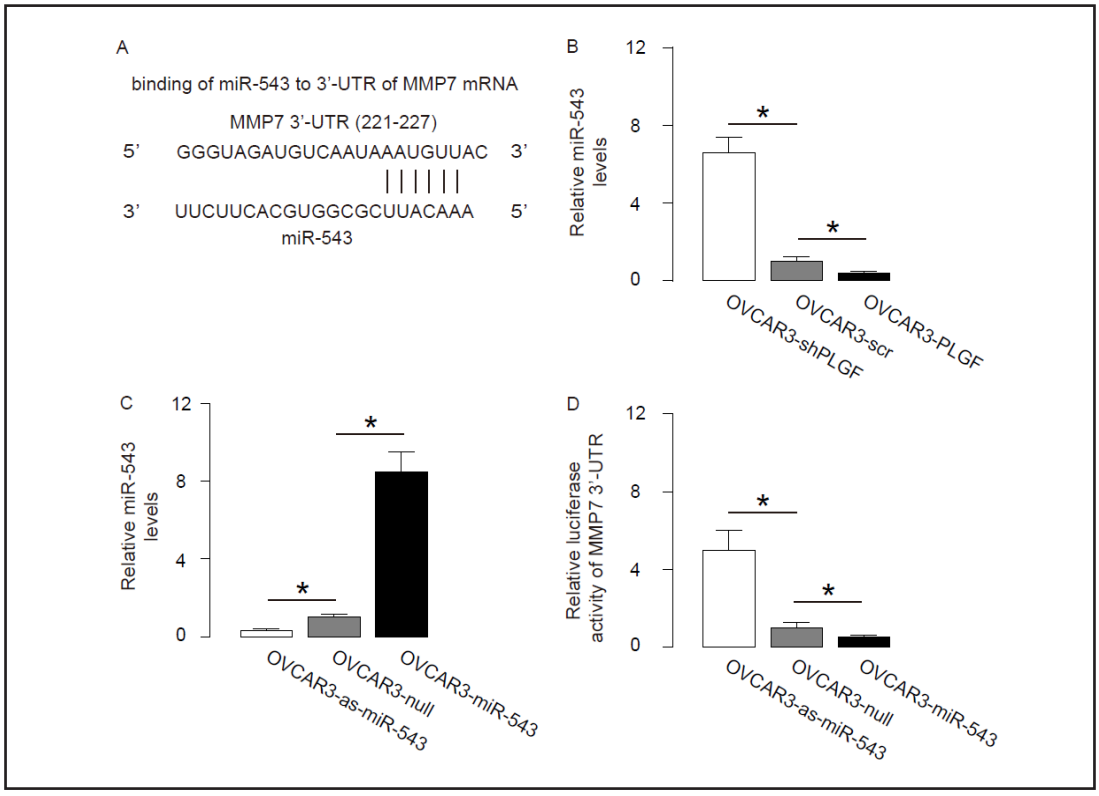

Fig. 3. PLGF suppresses miR-543, which targets 3'-UTR of MMP7 mRNA in OC cells. (A) Bioinformatics analyses of MMP7-target miRNAs, showing that miR-543 binds to 3'-UTR of MMP7 mRNA at 221th-227th base site. (B) The miR-543 levels in PLGF-modified OVCAR3 cells by RT-qPCR. (C) The OVCAR3 cells were transfected with either miR-543 or antisense for miR-543 (as-miR-543). OVCAR3 cells were also transfected with null sequence as a control (null). The miR-543 levels in miR-543-modified OVCAR3 cells were confirmed by RT-qPCR. (D). OVCAR3-miR-543, OVCAR3-null and OVCAR3-as-miR-543 cells were transfected with 1μg of MMP7-3'-UTR Luciferase-reporter plasmid, and the luciferase activities were quantified. *p <0.05. N = 5.

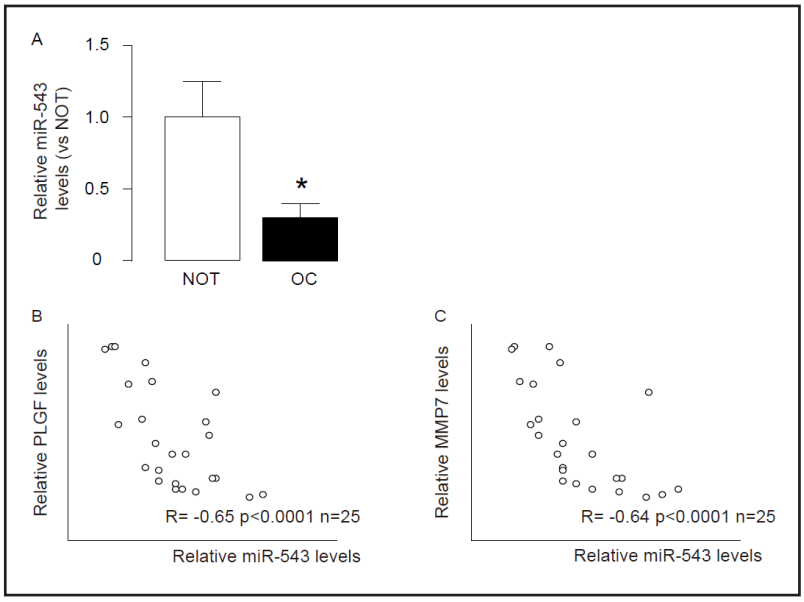

Fig. 4. OC expresses significantly lower levels of miR-543, which is inversely correlated with either PLGF or MMP7. (A) The miR-543 levels in the 25 OC specimens, by RT-qPCR. (B-C) A strong inverse correlation was detected between miR-543 and PLGF (B, R= -0.65; $p < 0.0001$), and between miR-543 and MMP7 levels (C, R= -0.64; $p < 0.0001$). *$p < 0.05$. N = 25.

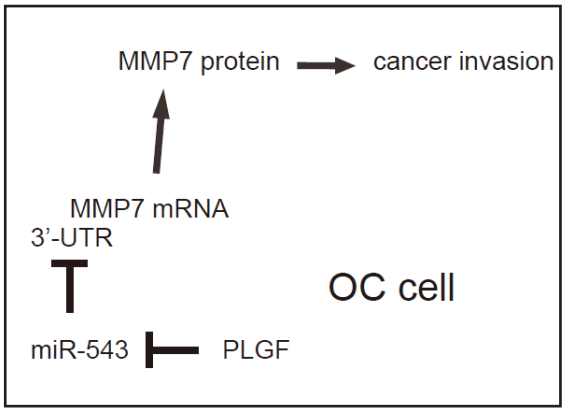

Fig. 5. A schematic model. MiR-543 may inhibit mRNA translation of MMP7 through 3'-UTR binding in OC. PLGF suppresses miR-543 to activate MMP7-mediated cancer invasion.

found that the luciferase activities in OVCAR3-as-miR-543 cells were significantly higher than the control, while the luciferase activities in OVCAR3-miR-543 cells were significantly lower than the control (Fig. 3D). These data suggest that miR-543 targets 3'-UTR of MMP7 mRNA to inhibit its translation in OC cells.

OC expresses significantly lower levels of miR-543, which is inversely correlated with either PLGF or MMP7

Finally, we examined the miR-543 levels in the 25 OC specimens, and found significantly lower levels of miR-543 in the OC specimens (Fig. 4A). Moreover, a strong inverse correlation was detected between miR-543 and PLGF (Fig. 4B, R= -0.65; $p < 0.0001$), and between miR-543 and MMP7 levels (Fig. 4C, R= -0.64; $p < 0.0001$). Together with our findings with OC cell line, these data suggest that miR-543 may inhibit mRNA translation of MMP7 through 3'-UTR binding in OC. PLGF suppresses miR-543 to activate MMP7-mediated cancer invasion (Fig. 5).

Discussion

Understanding the molecular basis underlying OC metastasis is potentially important for its efficient therapy. The growth and metastasis of OC have been shown to primarily depend on MMPs, including MMP7. Nevertheless, the molecular pathway controlling the activation of MMP7 in OC remains elusive.

Recently, MMPs have been shown to be regulated by vascular endothelial growth factor (VEGF) family members, e.g. VEGF-A and PLGF [19-26]. Of note, these angiogenic factors not only regulate angiogenesis, which is a process of development and of growth of new capillary blood vessels from pre-existing vessels, but also coordinate activation of other factors that are involved in cancer invasion, e.g. MMPs. Hence, the effects of VEGF-A and PLGF on cancer invasion are upon both cancer-associated vascularization and matrix-degradation-associated increases in invasiveness. The role of VEGF-A in tumor-related angiogenesis and invasion has been extensively studied. However, the role of PLGF in such settings is just recently put on table. Nevertheless, a series of recent studies have demonstrated a non-redundant role of PLGF in cancer-associated events, among which cancer invasion and metastases are the main results of PLGF expression.

In this study, we reported the causal relationship among PLGF, MMP7 and miR-543. PLGF and MMP7 appeared to be cancer promoters that contribute to increases in cancer invasiveness. We used several OC lines and got same results, suggesting that our findings should not be cell-line dependent. However, miR-543 seemed to a cancer suppressor that was silenced in OC, specifically by PLGF. Our study thus suggests that either suppression of PLGF or re-expression of miR-543 in OC cells may substantially prevent cancer metastases.

Disclosure Statement

The authors have declared that no competing interests exist.

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