

MicroRNA-133a-5p Promotes Angiogenesis of Endothelial Progenitor Cells via TRIM59/Id1 in Ovarian Cancer

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Research

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Abstract

Background

Endothelial progenitor cells (EPCs) play an important role in tumor angiogenesis and growth. In this study, we assessed the effect of MicroRNAs (miRNAs) on EPCs migration and angiogenesis and its signaling pathway in patients suffered from ovarian cancer (OC).

Methods

We cultured peripheral circulating EPCs derived from 32 OC patients and 20 healthy control subjects, respectively. The miRNA profiles of EPCs in ovarian cancer patients were compared with that in healthy control subjects, and aberrantly expressed miRNAs in both groups were identified via miRNA microarray and clustering analysis. Among these miRNAs, miR-133a-5p was considered as one of the most important miRNAs, which biological function in EPCs has been investigated. Bioinformatic analysis combined with knockdown and overexpression of miR-133a-5p were used to identify its target protein.

Results

An obviously downregulated expression level of miR-133a-5p has been seen in EPCs with ovarian cancer patients. Downregulated expression level of miR-133a-5p has been seen in ovarian cancer tissues and ovarian cancer cells (SKOV-3 and OVCAR-3). Downregulated of miR-133a-5p can increase TRIM59 expression, moreover, downregulated of miR-133a-5p further induce migration and angiogenesis via increase VEGF and Id1 in EPCs. MiR-133a-5p pro-angiogenesis would be diminished by TRIM59 knockdown.

Conclusions

The study found that miR-133a-5p was an important upstream factor regulated Id1/VEGF expression. Additionally, functional studies have revealed that TRIM59 was a direct target protein of miR-133a-5p, and TRIM59 silencing attenuated the role of miR-133a-5p in angiogenesis and Id1/VEGF expression. So we proposed that miR-133a-5p would be a new target for OC therapy.

Background

Ovarian cancer was ranked as the second lethal disease in gynecological malignancies. Currently, surgery and adjuvant chemotherapy still are the mainstream of treatment strategy [1]. In recent years, gene therapy as a novel approach has been used to treat OC, which shows promising therapy in the future. Many potential biomarkers in ovarian cancer have been detected by using gene chip and proteomics. Blood vessels are highly enriched in ovarian cancer, which are associated with poor prognosis, and anti-

angiogenic therapy is considered as a novel approach [2]. So OC has a tendency towards to invasion and metastasis, and it is also in resistant to conventional treatment.

More and more evidences have supported that EPCs could be a critical factor promoting progress of ovarian cancer by means of increase of angiogenesis and change of tumor microenviroment[3–5]. EPCs involve in angiogenesis in ovarian cancer, the pro-angiogenesis factors in which attracted widespread attention. Our previous study has indicated that over-expressed inhibitor of DNA binding/differentiation 1 (Id1) in EPCs could promote EPCs proliferation, migration, and adhesion[6–7]. Therefore, our present study focused on the factors related with regulation of angiogenesis through EPCs, which would be helpful for treatment of ovarian cancer.

Many previous reports have revealed that non-code microRNA (miRNA) play an important role in development of disease [8–13]. The miR-133 family has two members of miR-133a and miR-133b, which are found to specifically express in skeletal muscle and cardiac [9]. MiR-133a-5p belongs to the miR-133a cluster. Many studies have shown that miR-133 families are involved in regulating the proliferation and differentiation of various kinds of skeletal muscle cells [10–11]. MiR-133a can induce postmenopausal osteoporosis by repressing SLC39A1 expression and weakening osteogenic differentiation of hMSCs, and miR-133a is a potential biomarker for postmenopausal osteoporosis in circulating monocytes[12–13]. However, there were no articles that reported on the regulatory role of miR-133a-5p in EPCs migration and angiogenesis in ovarian cancer, and its underlying mechanism still was not clear.

Tripartite motif-containing 59 (TRIM59) functions as a ubiquitination ligase[14–15] or an adaptor protein[16–17], and plays important roles in various types of human cancers[14, 18, 19]. Recent studies showed that CDK5-dependent phosphorylation and nuclear translocation of TRIM59 promoted macroH2A1 ubiquitination and tumorigenicity[20]. However, it still remains unclear whether TRIM59 involve in regulation of angiogenesis and underlying mechanism in ovarian cancer. We predicted downstream target gene of miR-133a-5p using bioinformatic analysis, as a result, miR-133a-5p directly regulates TRIM59 that is an important factor in TRIM signaling pathway.

In this study, we assessed miR-133a-5p gene expression and its effect on EPCs migration and angiogenesis in ovarian cancer, and revealed the association between miR-133a-5p and TRIM59 also was defined.

Materials And Methods

Patients

All patients with ovarian cancer were histologically confirmed. All patients who were diagnosed with ovarian cancer had no additional malignant, other disease, such as inflammatory, ischemic disease, wounds, or ulcers that could affect the numbers of EPCs. Paired tissue samples were harvested from 25 patients with ovarian cancer who underwent primary cytoreductive surgery between March and August

2018. The study obtained the informed consent from all the participating patients and the approval from the Ethics Committee of the Cancer Hospital, Harbin Medical University.

Culture of EPCs

EPCs culture and identification have been performed, as described in our previous protocol[7], So that total mononuclear cells (MNCs) were isolated from 20 ml samples of human peripheral blood from patients with ovarian cancer by density gradient centrifugation with Histopaque-1077 (density 1.077 g/ml; Sigma Aldrich, St. Louis, MO, USA). MNCs were plated in 1 ml endothelial growth medium (EGM-2; Lonza, Basel, Switzerland) on fibronectin-coated (Sigma Aldrich) 24-well plates. After 24 h, non-adherent cells were discarded and adherent cells were cultured as above protocol. Medium was replaced every 2 days, thereafter, and each colony/cluster was followed up. At 7 days after culture, colony forming cells were identified by attached spindle-shaped cells. The adherent cells were incubated with DiI-acLDL (acLDL; Molecule Probes, Leiden, The Netherlands) and then fixed in 2% paraformaldehyde and counterstained with fluorescein isothiocyanate (FITC)-labeled lectin from *Ulex europaeus* agglutinin (UEA-1) (Sigma Aldrich).

Ovarian cancer cells culture

The IOSE80, SKOV-3 and OVCAR-3 cell lines were established in our laboratory. IOSE80, SKOV-3 and OVCAR-3 cells were grown in RPMI-1640 medium (Sigma, Oakville, ON, Canada) supplemented with 10% fetal calf serum (Hyclone Laboratories Inc., Logan, UT, USA). Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere in air.

miRNA microarray and clustering analysis

miRNA expression between EPCs with ovarian cancer patients and EPCs with healthy controls is unclear, gene expression profiling was examined using the PrimeView Human Gene Expression Array (Affymetrix), which includes 530,000 probes covering more than 36,000 transcripts and variants. Total RNA hybridization was conducted according to the manufacturer's instructions. Three repeats were performed from each sample to guarantee consistency of RNA hybridization. All subsequent technical procedures and quality controls were performed by Shanghai Genechem Co. Ltd, China. The arrays were scanned by a GeneChip Scanner 3000 (Affymetrix, inc, Santa Clara, CA, USA). GeneSpring GX software version 12.0 (Agilent Technologies, Palo Alto, CA, USA) was used to analyse the raw data obtained from each probe. Next, the data were normalized using the PLIER default protocols. Additionally, an unpaired t-test was applied to analyze the significantly differentially expressed genes. Hierarchical clustering analysis was used to reveal the relationship between significantly altered miRNAs in samples for each identified gene set with Euclidean distance and average linkage statistical methods.

Lentivirus transfection

The lentiviral vectors were purchased from Shanghai Genechem Company Ltd, China, which composed of the vectors pGCSIL-GFP which stably expressed siRNA and a marker (GFP-RFP fusion protein), and

pHelper1.0 (gag/pol element) and Helper2.0 (VSVG element). A non-silencing siRNA (5'-GCCTAACTGTGTCAGAAGGAA-3') was used as the negative control (NC). The siRNA sequences targeting miR-133a-5p gene were 5'-CGTAATTCCGATTATACGTCCATC-3' and 5'-GTGATGCGATTATGCTCCCT-3'. The siRNA sequences targeting TRIM59 gene were 5' -AGTATTACCCTTCATACCATCAA-3' and 5'-GTCATGCGCTTTTCCAGCCC-3'. The overexpression sequences targeting miR-133a-5p gene were 5'-TGTGATTACCATGATCCTCCCTTG-3' and 5'-ATCAAGGGGTAATTCTCTCT-3'. The overexpression sequences targeting TRIM59 gene were 5' -TATGTTAGCATGCATGCTATCAGA-3' and 5'-GACTTCCTCTATGCGAGTCG-3'. All the EPCs were planted on a six-well plates at 5×10^4 cells per well and incubated, respectively. Appropriate volumes of lentivirus were added to the cells according to the recommendation of manufacturer, when cell fusion reached to 60%.

Luciferase assays

A mutant construct of TRIM59 3'-UTR or TRIM22 3'-UTR was obtained by introducing a mutation into the seven nucleotides (CCCGUAA) of the seed region for miR-133a-5p. The miR-133a-5p target sequence in the coding region of TRIM59 or TRIM22 was amplified by PCR and cloned into GV143 that contained a firefly luciferase reporter gene. Wild-type TRIM59/TRIM22 3'-UTR or mutant TRIM59/TRIM22 3'-UTR and the empty 3'-UTR vector were cotransfected into HEK293 cells, with Renilla luciferase vector transfection as reference. After incubation for 48 hrs, the cells were harvested and assayed for Renilla and firefly luciferase activities using the dual luciferase reporter assay system (Promega). The relative luciferase activities were calculated by normalizing to Renilla luciferase. Cells were transfected with the/an empty 3'-UTR vector as a negative control (NC).

Transwell Migration Assay

At 7 days after incubation, the cultured medium were removed and replaced with EBM-2 without any supplements cells were cultured for 12 hours for the migration assay. EPCs migration was evaluated using a transwell migration assay. Briefly, 5×10^4 cells were suspended in 100 μ L of EBM-2 supplemented with 0.1% BSA and placed in the upper chamber of an 8.0-mm pore size transwell (Costar, Cambridge, MA). 600 μ L of the final dilution was placed in the lower chamber. After incubating for 6 hours at 37°C in 5% CO₂, the cells that had not migrated were removed from the upper surface of the filters using cotton swabs and those that migrated to the lower surface of the filters were fixed in methanol and stained with Giemsa's Stain Solution. Migration was determined by counting the cells with a microscope. Five visual fields were randomly chosen for each assay. The average count of the migrating cells in these 5 fields was taken as the migrated cells of the each group.

In vitro tube formation

In vitro tube formation assay was performed using the Matrigel basement membrane matrix (BD Biosciences). 1 ml/well Matrigel, kept on ice, was placed in 4-well culture plates. The plates were then incubated at 37°C for 30 min to allow Matrigel to solidify. About 4×10^4 EPCs were cultured on the

preplated Matrigel and incubated for 48h. To quantitate the in vitro angiogenesis, the average tubular area (enclosed spaces) and the average of tube area per total field area in 5 serial microscopic fields were calculated. The tube area and tube length were expressed as μm^2 and μm using image-analyzing software and AxioVision Version Rel 4.8 Software.

Real-time quantitative polymerase chain reaction(qPCR)

Total RNA was extracted from EPCs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was quantified by absorption at 260 nm. The isolated RNA was then DNase-treated and reverse-transcribed according to the manufacturer's recommended protocols. Briefly, miRNAs were reversely transcribed using the Primescript Reverse Transcription kit, miScript syBRGreen PCR kit and miScript Primer Assays according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed using an ABI 7500 sequence detection system. The Cycling parameters were 2 min. at 50°C and 10 min. at 95°C, followed by a total of 40 cycles of 15 sec. at 95°C and 1 min. at 60°C. All of the reactions were performed in triplicate. The gene expression $\Delta\Delta\text{CT}$ values of miRNAs were calculated by normalizing U6 to an internal control.

Western blot analysis

EPCs were collected in sample buffer and then incubated in lysis buffer and protease inhibitors for 30 min. Kept on ice. Next, the supernatants were collected following centrifugation at $1.3 \times 10^4 \times g$ at 4°C for 15 min. Total cell proteins were extracted using the mammalian protein extraction reagent including halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentrations were measured by BCA protein assay kit (Thermo Fisher Scientific) and equal amount of proteins were subjected to SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After being blocked with 5% nonfat milk, the membranes were incubated with primary antibodies against VEGF, Id1, TRIM59 and Actin (Santa Cruz), respectively, and followed by incubation with the HRP-conjugated secondary antibodies. The signals were detected using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA)

Statistical analysis

All experiments were repeated at least three times. Data were shown as means \pm standard deviation (S.D.). P- values less than 0.05 were considered to be statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001). Statistical comparison between groups was performed using Student's t-test.

Results

Identification of differentially expressed miRNAs in EPCs

To identify miRNAs expression in EPCs with ovarian cancer patients, we compared the profiles of differentially expressed miRNAs in EPCs from ovarian cancer patients and healthy controls through

analysis of microarray. Hierarchical clustering displayed a significantly increased expression in six miRNAs (miR-138, miR-365, miR-156, miR-141-3p, miR-100, miR-99p), whereas, other six miRNAs (miR-150, miR-132, miR-133a-5p, miR-127, miR-145, miR-450-5p) appeared a clearly decreased expression (Fig. 1A). To confirm the findings of the microarray analysis, we detected the miRNAs expressions that ranked first four up- and down-regulated in q-PCR, and discrepancies in the expression of each of these miRNAs were confirmed (Fig. 1B).

miR-133a-5p expression and effects of miR-133a-5p on EPCs migration and angiogenesis

Due to the association of miRNAs in EPCs with ovarian cancer patients. It may be involved in the regulation of angiogenesis in EPCs. Furthermore, our previous study has demonstrated that Id1 expression was up-regulated in EPCs with OC patients, compared with healthy controls. Id1 can promote angiogenesis by regulating VEGF during the development of EPCs. So, we constructed luciferase reporter plasmids consisting of Id1 and VEGF wild-type or mutant 3'-UTR sequences, and cotransfected with each of the above mentioned four upregulation miRNAs mimic and four downregulation miRNAs siRNA lentirival. As shown in Figure 2A and B, the activities of VEGF and the Id1 reporter showed a significant increase by transfected miR-133a-5p siRNA lentirival, however, miR-365 mimic resulted in a decrease of VEGF reporter activity, but we did not find significant change in activities of reporter transfected with other miRNAs. According to these findings, we proposed that miR-133a-5p may regulate Id1/VEGF to induce angiogenesis of EPCs.

To investigate miR-133a-5p expression in EPCs from OC patients, we detected the expressions of miR-133a-5p in EPCs of 20 OC patients and 20 healthy controls using qPCR, respectively. The results showed that miR-133a-5p expression decreased by 6.17-fold in EPCs from ovarian cancer patients, it was consistent with the result that miR-133a-5p expression decreased by 6.51-fold in microarray analysis (Fig. 2C). At the same times, the levels of miR-133a-5p expression in ovarian cancer tissues were detected, the results showed a decreased expression, compared with that in normal ovarian tissues (Fig.2D). The same results in OC cells (SKOV-3, OVCAR-3) and normal ovarian cells (ISOE80) were also found (Fig.2E).

We evaluated the influence of miR-133a-5p on EPCs migration and angiogenesis using transwell and matrigel. Knockdown and overexpression of miR-133a-5p not only can enhance and decrease obvious cell migration, respectively (Fig. 3A, 3C), but also they can increase and decrease EPCs angiogenesis, respectively (Fig. 3B, 3D, 3E). To verify whether miR-133a-5p regulated Id1 and VEGF as an upstream factor. MiR-133a-5p knockdown lentirival was transfected into the ovarian cancer EPCs. WB analysis supported that the levels of VEGF and Id1 protein were significantly increased (Fig.3F and 3G). These findings confirmed that miR-133a-5p could induce angiogenesis of EPCs through upregulation on VEGF and Id1.

TRIM59 is upregulated in EPCs of ovarian cancer patients and shows association with EPCs migration and angiogenesis

To clarify other genes and pathways mediated by miR-133a-5p, including potential target genes that miR-133a-5p directly regulated, we did bioinformatic analysis (www.targetscan.org). TRIM and mitogen-activated protein kinase (MAPK) signaling pathways were most closely related with miR-133a-5p. As a result, TRIM and MAPK, as potential signaling pathway of miR-133a-5p, were used to further investigate. MiR-133a-5p knockdown expression did not obviously interfere with the mRNA levels of MAP3K13, MAPK2 or MAPK11P1L in EPCs; however, miR-133a-5p knockdown expression significantly increased the mRNA levels of both TRIM59 and TRIM22 (Fig. 4A).

To further explore the possible function of TRIM59 and TRIM22, we constructed luciferase reporter plasmids consisting of TRIM59 or TRIM22 wild-type or mutant 3'-UTR sequences. We found that only activity of the TRIM59 reporter was obviously increased following cotransfection of miR-133a-5p knockdown lentirival, whereas, activity of TRIM22 reporter was not found significant difference (Fig. 4B). These findings uncovered that miR-133a-5p targeted to the 3'-UTR sequence of TRIM59, but not TRIM22.

To determine whether TRIM59 expression would be different in EPCs between OC patients and healthy controls, we did Western blotting assay. The results showed an obvious increase in TRIM59 expression in EPCs of OC patients, compared with that in healthy controls (Fig. 5A-B). Furthermore, we infected with TRIM59 overexpression and knockdown lentirival into the human ovarian cancer EPCs, respectively. We assessed the effects of TRIM59 overexpression and knockdown on EPCs migration and angiogenesis by transwell and matrigel, as consequence, migrated cells were obviously increased and decreased respectively. TRIM59 overexpression caused an obvious cell migration. TRIM59 knockdown caused an decreased cell migration (Fig. 5C, 5E). Furthermore, overexpression of TRIM59 induced EPCs angiogenesis. Knockdown of TRIM59 induced decreased EPCs angiogenesis (Fig. 5C, 5F, 5G). Taken together, our results demonstrated that decreased miR-133a-5p directly up-regulates TRIM59 that can promote migration and angiogenesis of EPCs .

miR-133a-5p regulated EPCs angiogenesis through TRIM59, Id1 and VEGF

To clarify the effect of miR-133a-5p on angiogenesis through TRIM59 in EPCs, we investigated the impact of TRIM59 knockdown on effects of miR-133a-5p in angiogenesis. Matrigel assay showed that TRIM59 knockdown compromised the effects of decreased miR-133a-5p on increasing angiogenesis (Fig. 6A-C).

Given these roles of Id1 and VEGF in the process of EPCs angiogenesis, as well as upregulation in ovarian cancer, we investigated whether TRIM59 had effects on Id1 and VEGF expressions by mediated miR-133a-5p. TRIM59 knockdown attenuated the functions of decreased miR-133a-5p on both Id1 and VEGF expressions (Fig. 6D and E). These results suggested that miR-133a-5p induced angiogenesis of EPCs may be mediated by TRIM59 signaling pathway in the progress of ovarian cancer. It also confirmed the hypothesis that TRIM59 may be a direct target of miR-133a-5p in ovarian cancer.

Discussion

In our previous study, we have confirmed the potential effect of Id1 in EPCs on ovarian cancer metastasis and angiogenesis development. In the present study, we identified different miRNAs related to angiogenesis of EPCs using μ Paraflo Microfluidic Array Technology. Because the balance of VEGF and Id1 is an important factor in mediating angiogenesis [21], so miRNA affected activities of VEGF and Id1 was detected. Our results revealed that miR-133a-5p knockdown could significantly increase activities of VEGF and Id1, it also suggested that miR-133a-5p could regulate angiogenesis of EPCs on upstream of VEGF/Id1. We found that miR-133a-5p expression level was significantly lower than that in normal ovarian tissues as well as in SKOV-3 and OVCAR-3 cells.

There are a lot of studies regarding association between miRNA and ovarian cancer [22-25]. Our study identified the different miRNAs related to angiogenesis that contributed to metastasis of ovarian cancer using high throughput technology. With regarding function of miR-133a-5p, the recent study has pointed that miR-133a-5p inhibits androgen receptor (AR)- induced proliferation in prostate cancer cells via targeting Used in Sarcoma (FUS) and AR[26]. Additionally, circP4HB enhances EMT and metastatic disease through miR-133a-5p sequestration, leading to upregulation of vimentin[27]. To further investigate association of miR-133a-5p with angiogenesis of EPCs in ovarian cancer, miR-133a-5p expression was knockdown by infected lentivirus vector with miR-133a-5p-siRNA, it resulted in increase of migration and angiogenesis of EPCs. At the same time, the levels of VEGF and Id1 gene expression also were up-regulated. Taken together, our study supported that miR-133a-5p could increase EPCs angiogenesis and regulate microenvironment in ovarian cancer.

Based on the results of bioinformatic analysis, we proposed that miR-133a-5p may be associate with TRIM59 signaling pathway. In our study, knockdown of miR-133a-5p induced up-regulation of TRIM59 expression, it suggested that TRIM59 is a target gene of miR-133a-5p, further, miR-133a-5p regulate TRIM59 signaling pathway, which exert a key role in angiogenesis of EPCs and OC metastasis. Next, the study demonstrated that TRIM59 expression was reversely associated with miR-133a-5p expression level in EPCs with OC patients. The effect of overexpression of TRIM59 on EPCs migration and angiogenesis also supported our hypothesis. In present study, underexpression of miR-133a-5p altered Id1 and VEGF protein levels. As a result, miR-133a-5p-induced angiogenesis involved in the TRIM59-mediated regulation in Id1 and VEGF protein levels.

Conclusion

Our findings revealed the down-regulation of miR-133a-5p in EPCs of OC patients, TRIM59 as a target gene of miR-133a-5p that regulates EPCs angiogenesis. Furthermore, miR-133a-5p may also suppress TRIM59, and it lead to Id1/VEGF mediated migration and angiogenesis of EPCs in the patients with ovarian cancer. Based on these findings, the interaction between miR-133a-5p and its target gene TRIM59 should be further studied as a predictor or therapeutic target that could improve therapeutic outcomes in ovarian cancer.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study obtained the informed consent from all the participating patients and the approval from the Ethics Committee of the cancer Hospital, Harbin Medical University. All individuals participating in this study provided written informed consent.

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Consent for publication

Not applicable.

Availability of data and materials

The data used and analyzed during this study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YJS conceived of the study and drafted the manuscript. LCT and JW carried out the molecular biological studies and performed the statistical analysis. YW and HYW participated in its design and helped to draft the manuscript. MY and YZ collected the patient information. HLL helped to revise the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

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Figures

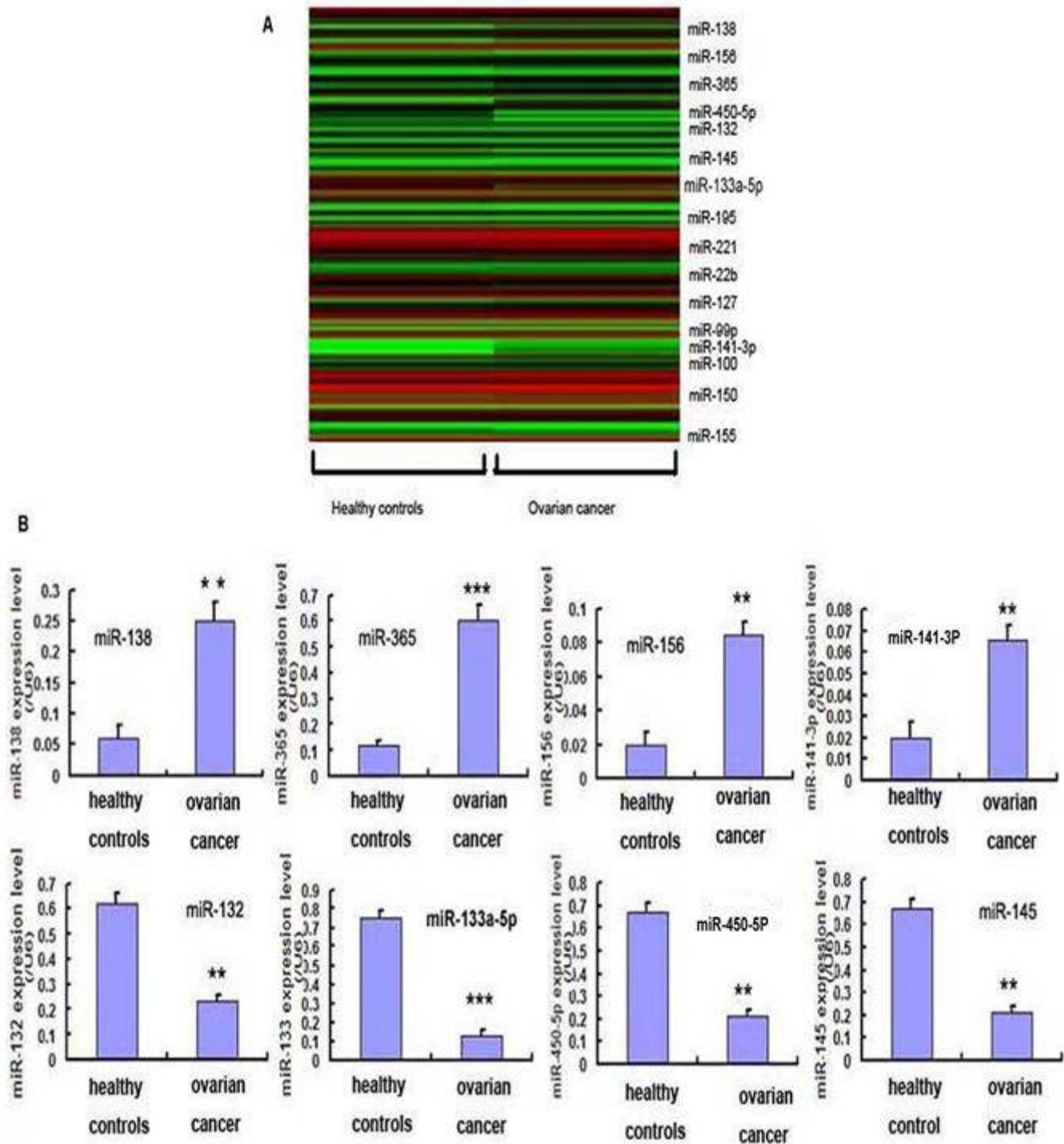


Figure 1

Differentially expressed miRNAs in EPCs with ovarian cancer patients and healthy controls. (A) Hierarchical clustering showing the profiling data of up- or down-regulated miRNAs in EPCs with ovarian cancer patients and healthy controls using microarray analysis. Red indicates higher relative expression, while green indicates lower relative expression. (B) Expression of first four up-regulated miRNAs (miR-138, miR-365, miR-156, miR-141-3p) and first four down-regulated miRNAs (miR-132, miR-133a-5p, miR-450-5p, miR-145)

miR-145) in EPCs from OC patients and healthy controls were identified by qPCR. The data are shown with the means \pm S.D. of three independent experiments (** $P < 0.01$; *** $P < 0.001$).

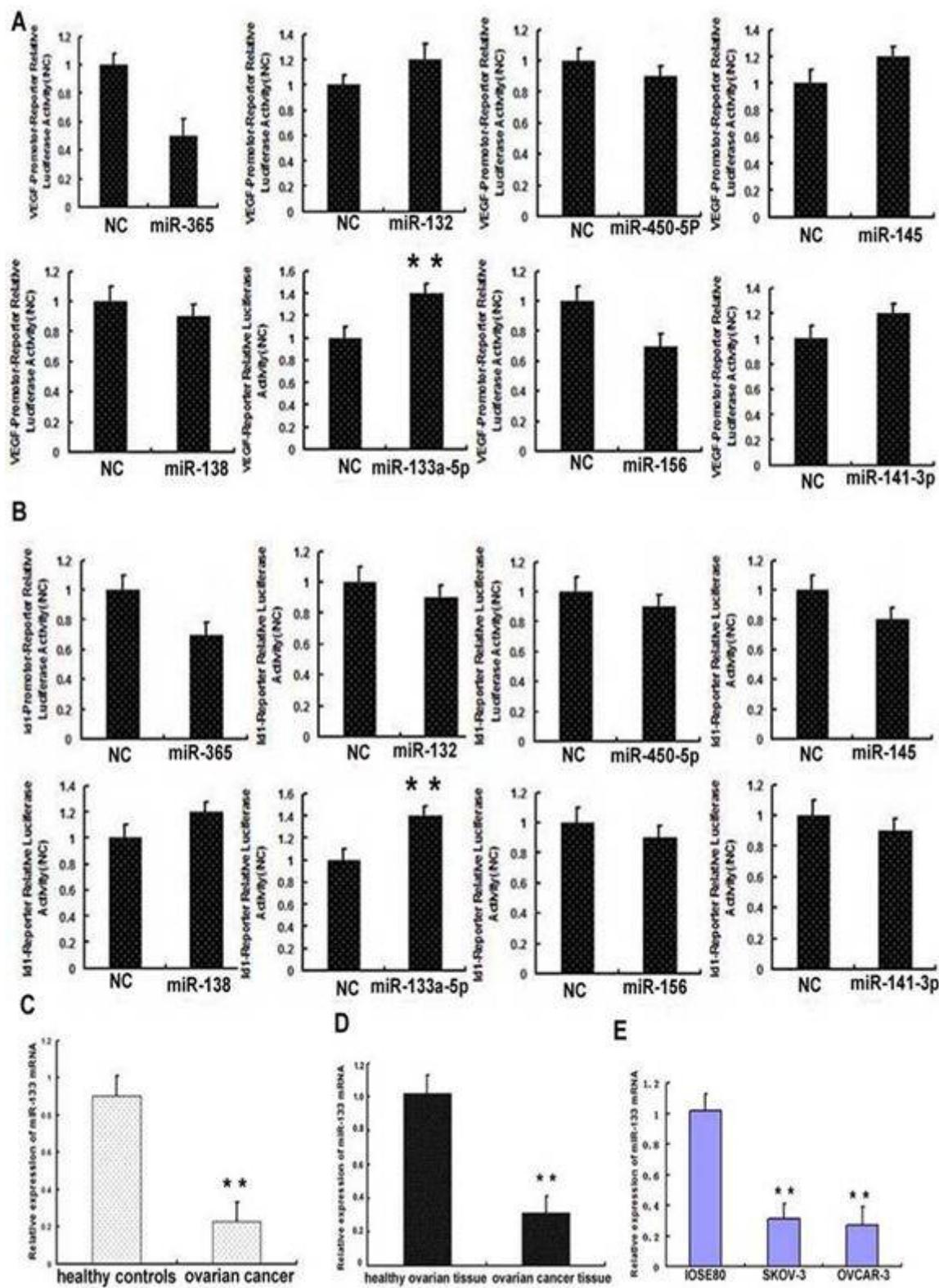


Figure 2

miR-133a-5p altered VEGF and Id1 expression in EPCs with ovarian cancer patients. (A and B) Effects of first four up-regulated and down-regulated miRNAs on Id1 and VEGF transcriptional activities. miRNAs or empty vector (NC) lentivirus was cotransfected into HEK293 cells with the Id1-promoter-reporter or the VEGF-reporter. Renilla luciferase vector as reference was transfected. (C) Expression of

miR-133a-5p in EPCs with ovarian cancer patients and healthy controls. Similar results were found in ovarian cancer patients tissues and ovarian cancer cells (D and E) .The data represent the means \pm S.D. of three independent experiments (**P < 0.01).

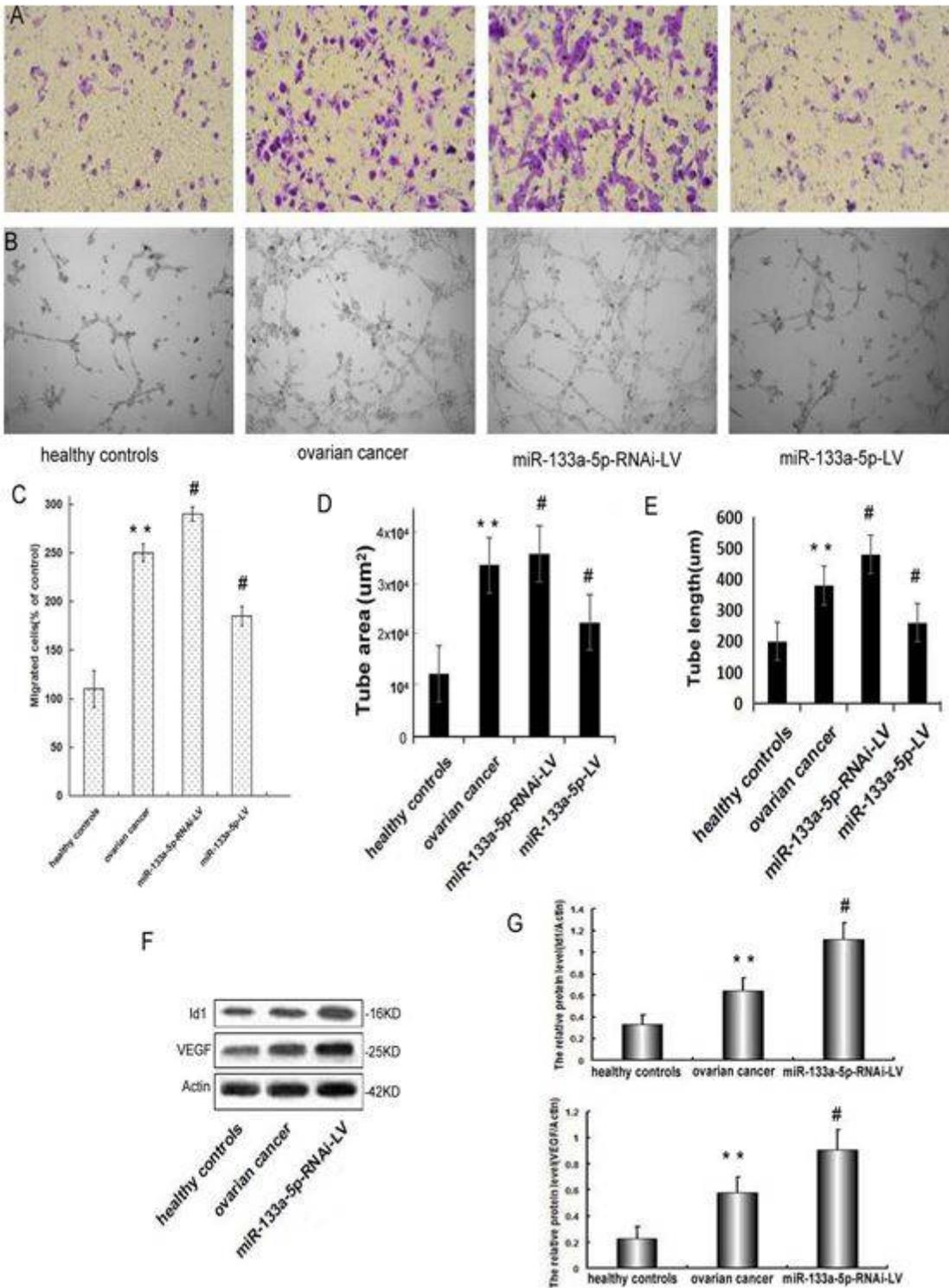


Figure 3

Effects of miR-133a-5p expression on the migration and angiogenesis of EPCs. (A) The cell migration was assessed by transwell in EPCs after transfection with miR-133a-5p knockdown and overexpression lentirival. The cell migration representative images are shown. (B) The cell angiogenesis was assessed by matrigel in EPCs after transfection with miR-133a-5p knockdown and overexpression lentirival. The cell

angiogenesis representative images are shown. (C) Accumulated data showing EPCs migration function. Comparison of tube area (D) and tube length (E) are shown. (F and G) Typical Western blot images showing protein expression of VEGF and Id1 (β -actin is shown as a housekeeping control). The graph showing the relative VEGF and Id1 protein levels normalized to actin. The results are expressed as the mean \pm S.E. (** $p < 0.01$ vs. healthy controls; # $p < 0.01$ vs. ovarian cancer).

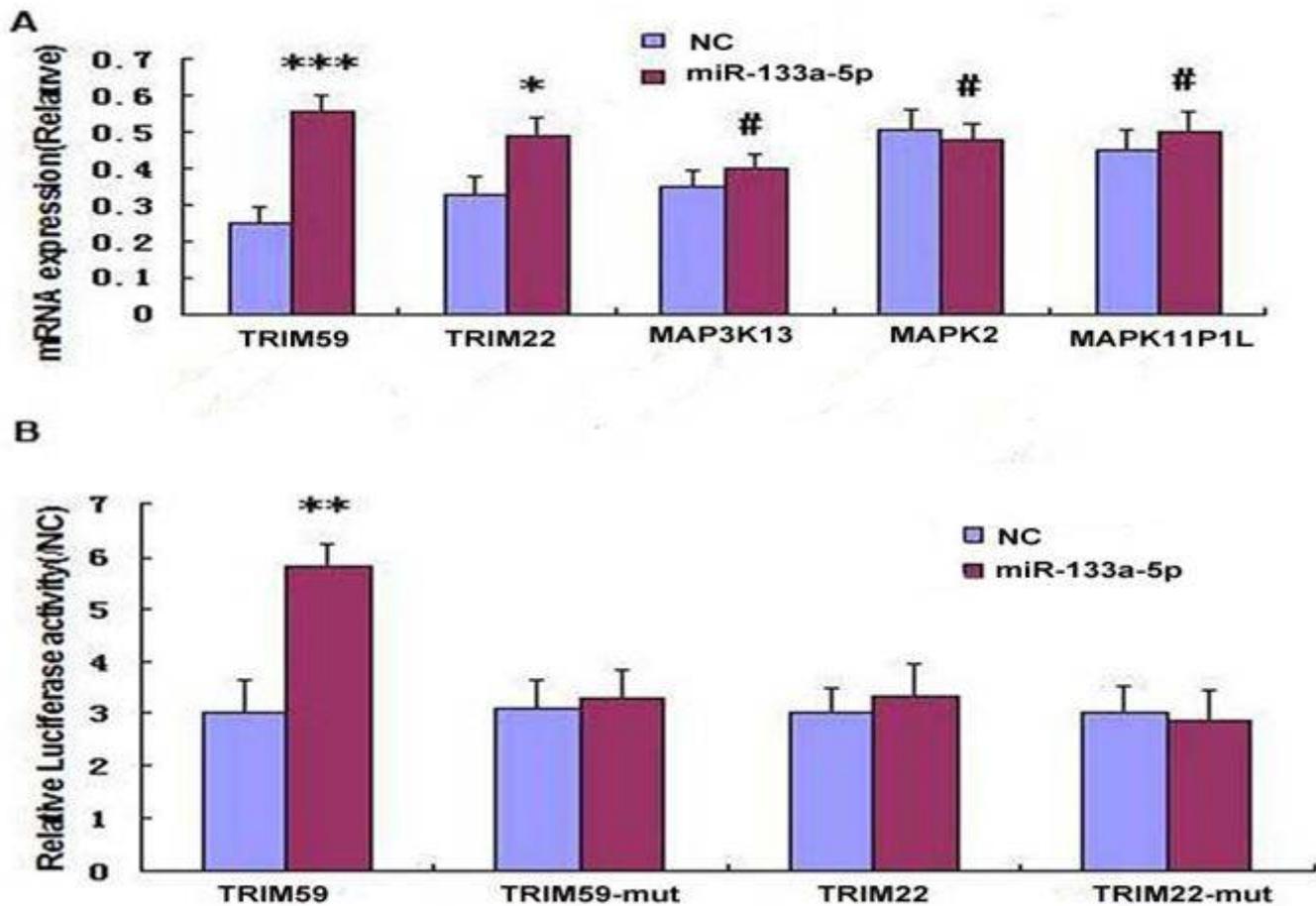


Figure 4

TRIM59 was identified as an miR-133a-5p target gene that is upregulated in EPCs with ovarian cancer patients. (A) EPCs were treated with miR-133a-5p-RNAi-LV or NC-LV (NC). At 36 hrs after transfection, expressions of genes at mRNA levels in the TRIM and MAPK pathway were analyzed by q-PCR. (B) TRIM59, TRIM59 mut, TRIM22 and TRIM22 mut 3' -UTR luciferase reporters with miR-133a-5p-RNAi-LV or NC-LV (NC) were cotransfected into HEK293 cells. At 36 hrs posttransfection, the luciferase activity was detected by means of the Dual-Luciferase reporter assay system. The relative luciferase activity was normalized using Renilla luciferase activity. The data represent the means \pm S.D. of three independent experiments (** $P < 0.01$; ** $P < 0.01$; * $P < 0.05$; # $P > 0.05$ vs. NC)

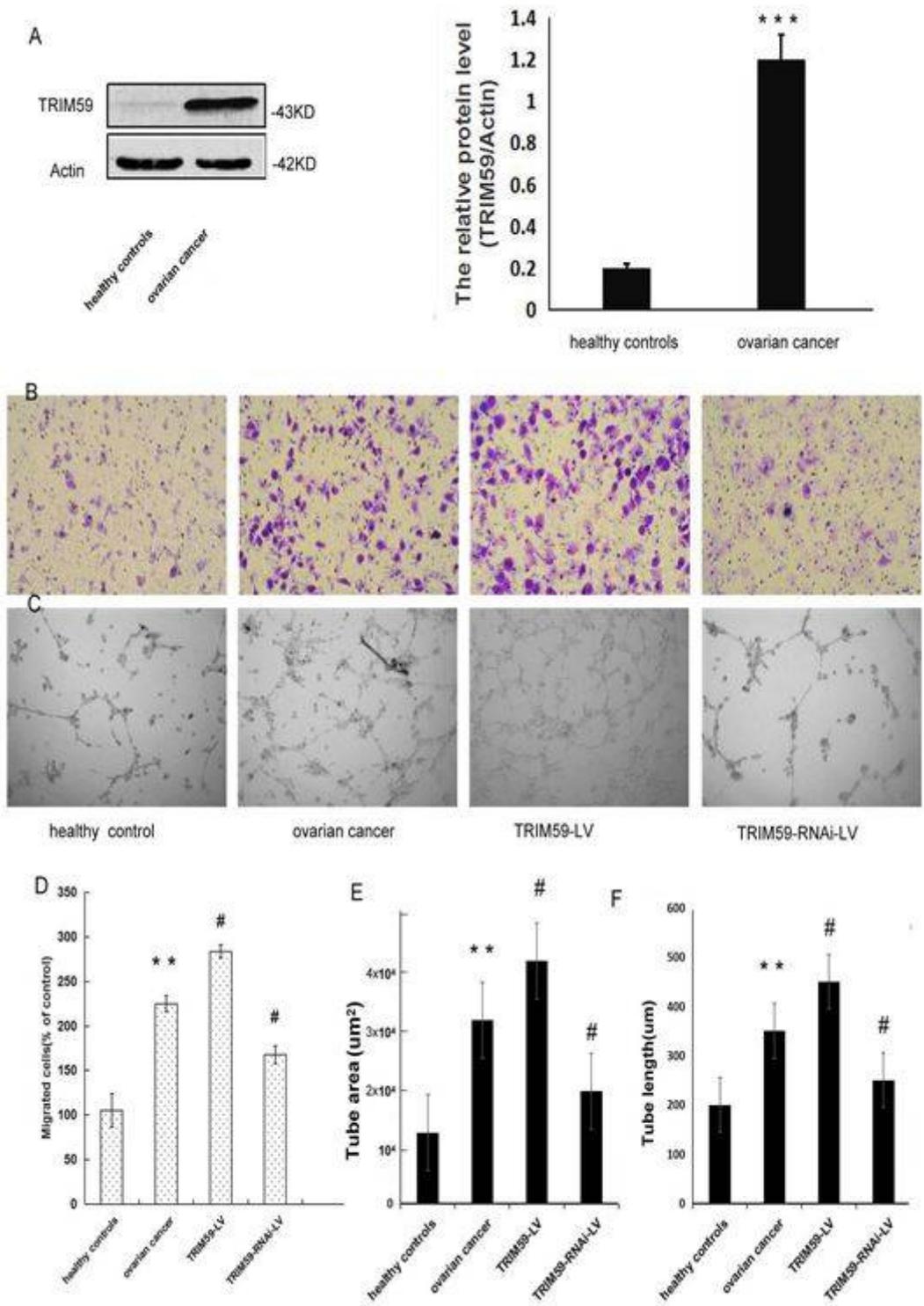


Figure 5

Effects of TRIM59 expression on the migration and angiogenesis of EPCs. (A) The relative expression level of TRIM59 protein in EPCs with ovarian cancer patients relative to healthy controls was assessed using Western blotting, actin protein was used as a loading control. The data are shown using the means \pm S.D. of three independent experiments (***)P < 0.001 vs. healthy controls). (B) EPCs were transfected with TRIM59 overexpression and knockdown lentirival, respectively. The cell migration was detected by

transwell following 36 hrs. The pictures of cells migration are shown. (C) EPCs were transfected with TRIM59 overexpression and knockdown lentirival, respectively. After 36 hrs, the cell angiogenesis was detected by matrigel. The pictures of cells angiogenesis are shown. (D) Accumulated data showing EPCs migration function. Comparison of tube area (E) and tube length (F) are shown. (**P < 0.01 vs. healthy controls; # P < 0.01 vs. ovarian cancer).

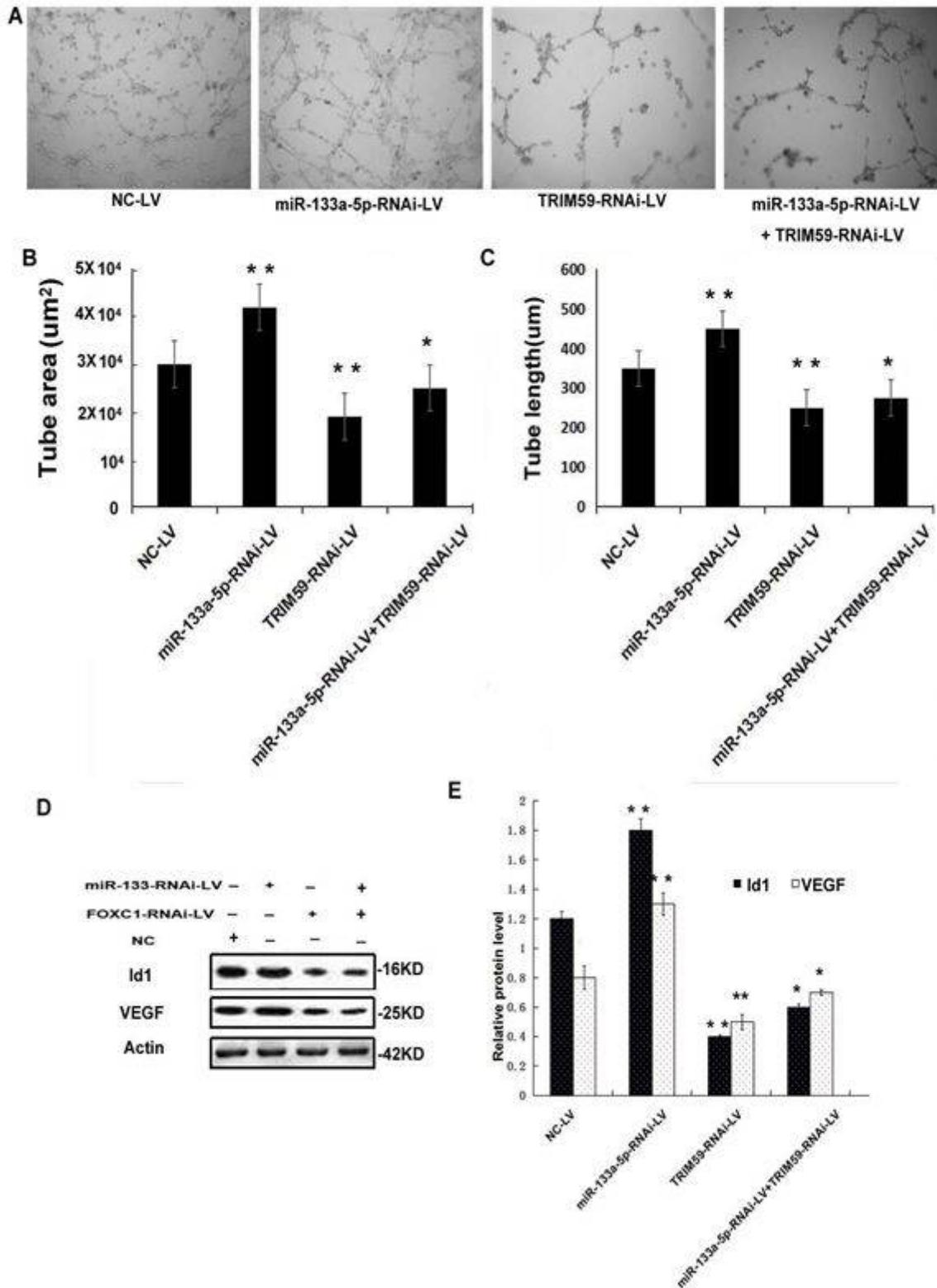


Figure 6

TRIM59 downexpression affected the miR-133a-5p regulated signaling pathway for angiogenesis in EPCs. EPCs were treated with NC-LV(NC), miR-133a-5p-RNAi-LV, TRIM59-RNAi-LV, miR-133a-5p-RNAi-LV + TRIM59-RNAi-LV. (A) At 36 hrs posttransfection, the EPCs angiogenesis were analysed by matrigel. Comparison of tube area (B) and tube length (C) are shown. (**P < 0.01 vs. ovarian cancer). (D and E)

The expressions of VEGF and Id1 at protein level were detected by Western blot analysis post transfection. Relative VEGF and Id1 protein levels were normalized to the levels of actin. The data are represented with the means \pm S.D. of three independent experiments. (*p < 0.05; **P < 0.01 vs. NC).