

## Original Article

# PDGF-Rb and microRNA-329 are associated with endometrial dysfunction via targeting CD146

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**Abstract:** It is believed that endometrial miRNAs contribute to the etiology of endometrial dysfunction, however, the mechanisms remain unclear. Here we collected endometrial tissues from infertility patients with different thickness and characterized the miRNA expression profiles of these three groups. MiR-329 was dramatically down-regulated in infertility patients with endometrial hyperplasia. In addition, we found that miR-329 overexpression could inhibit CD146 expression in ESCs, which is a marker and is mainly expressed in the cytoplasm of ESCs. Moreover, bioinformatics analysis suggested that miR-329 was a regulator of CD146. Importantly, up-regulation of miR-329 in ESCs decreased cell proliferation, migration and invasion, and the underlying mechanism was mediated, at least partially, through the suppression of CD146 expression. Furthermore, PDGF increased cell viability, migration and invasion in a dose-dependent manner, PDGF-Rb antagonist, imatinib, could reverse PDGF-induced ESCs dysfunction through the activation of PDGF/PDGFR-dependent signaling pathways. In conclusion, PDGF-Rb could play an important role in endometrial hyperplasia, and miR-329 via targeting CD146 associated with endometrial dysfunction and might be a potential therapeutic target against endometrial hyperplasia.

**Keywords:** micorRNA-329, endometrial hyperplasia, CD146, PDGF-Rb

## Introduction

In the last two decades, with the development of assisted reproductive technology, patients have got multiple opportunities for embryo transfer, which makes the role of the receptivity of endometrium more important in pregnancy [1]. It is well-known that both thinned and thickened endometrium can lead to reduce the endometrial receptivity [2, 3]. Moreover, thickened endometrium indicates an increased risk of endometrial carcinoma or other tissue pathological changes, such as hyperplasia or polyp.

CD146, an adhesion molecule belonging to the immunoglobulin superfamily, has been identified as a melanoma-specific cell adhesion molecule [4, 5]. A growing body of evidence suggests that CD146 may be involved in tumor development, such as hepatocellular carcinoma, melanoma, epithelial ovarian cancer and breast cancer [4, 6]. In endometrial stem cells (ESCs), CD146 is observed and is a marker of ESCs [7]. However, the molecular mechanisms of CD146 contribute to the induction of endometrial hyperplasia remains to be determined.

MicroRNAs (miRs) are endogenous non-coding RNAs and single-stranded RNA molecules of ~22 nucleotides in length that serve as important post-transcriptional gene regulators [8]. The key features of miRs control cell proliferation and differentiation of various cell types. MiRNA-329 can inhibit cell proliferation in human glioma and liver cancer cells, and suppress angiogenesis by targeting CD146 in human umbilical vein endothelial cells (HUVECs) [9, 10]. However, no study to date has reported on miRNA-329 and CD146 expression in endometrium. In the present study, we compiled data from patients diagnosed with endometrial hyperplasia to elaborate the pathophysiological mechanism mediated by miRNA-329 via targeting CD146.

## Materials and methods

### *Patients and specimens*

Sixty women were diagnosed with infertility by laparoscopy or laparotomy, according to the revised American Society of Reproductive Me-

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dicine Classification. Sixth endometrial tissues were collected from the Center of Reproductive Medicine, Yuhuangding Hospital Affiliated to Qingdao University between Jan 2012 and June 2014 (Yantai, China). Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics Committee of the Yuhuangding Hospital Affiliated to Qingdao University, China. Human were divided into three groups by the thickness of endometrium: (1) thinning endometrium (TEM group, n = 20), (2) normal endometrial thickness (NC group, n = 20), (3) endometrial hyperplasia or proliferation (HEM or PEM group, n = 20).

### *Cell culture*

Endometrial stem cells (ESCs) were obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China). ESCs and HEK293 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) with 1 g/liter glucose and 10% FCS at 37°C in a humidified incubator (Thermo, USA), 5% CO<sub>2</sub>, 95% air atmosphere.

### *Cell viability, motility, migration and invasion assay*

ESCs in FBS-free medium were treated with theaflavins for 48 h, 10 µL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (MD SpectraMax M5, USA) according to the manufacturer's instructions. Migration assay used Transwell inserts (Costar, NY, USA; 8-mm pore size) in 24-well dishes. For invasion assay, filters were precoated with 30 µL Matrigel basement membrane matrix (BD Biosciences, Bedford, MA, USA) for 30 min. The following procedures were the same for wound-healing, migration and invasion assays. After 48 h, cells were harvested and seeded to Transwell at  $1 \times 10^4$  cells/well in serum-free medium, and then incubated for 24 h at 37°C in 5% CO<sub>2</sub>. Cells were fixed in 4% formaldehyde for 5 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of filters were removed with cotton-tipped swabs, filters washed with PBS. Cells on the underside of filters were examined and counted under a microscope.

### *Oligonucleotides and transfection*

siRNA duplexes targeting coding sequence (CDS) positions 410 to 428 for CD146 or CDS positions 1562 to 1580 for green fluorescent protein (GFP) were synthesized by Invitrogen. The miR-329 mimics, 2'-O-methyl antisense oligonucleotide targeted toward miR-329 (anti-miR-329) and negative control (anti-miR ctrl), were purchased from GenePharma.

### *Luciferase reporter gene activity assay*

The 3'UTR of CD146 gene containing the predicted target sites for miR-329 was obtained by PCR amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion, Austin, USA). HEK-293 cells were co-transfected with 0.1 µg of luciferase reporters containing CD146 3'UTR and miR-329 mimics by Lipofectamine 2000 (Invitrogen, Carlsbad, USA). We harvested the cell lysates after 48 h transfection and measured the luciferase activity with a dual luciferase reporter assay kit according to manufacturer's instruction.

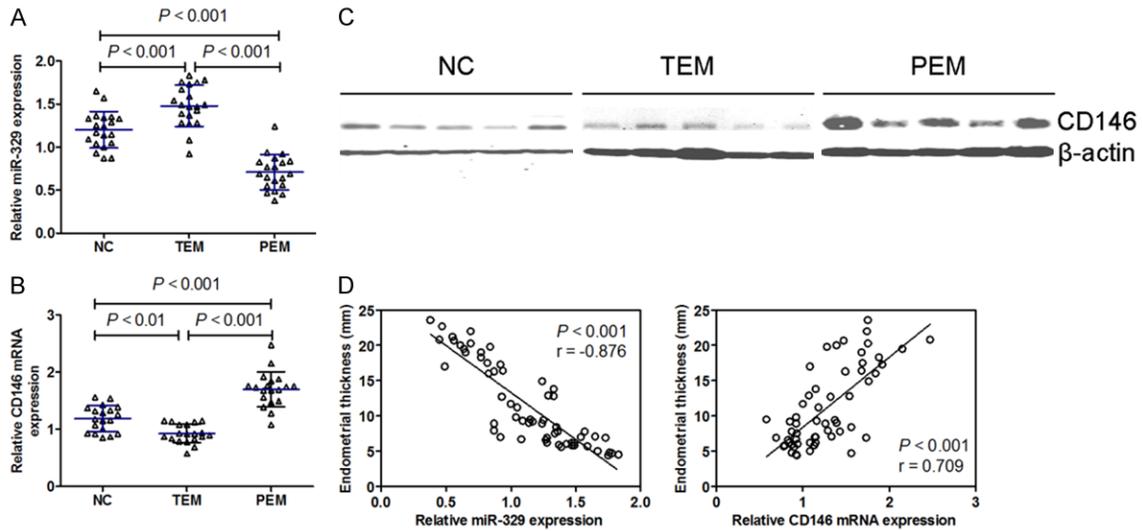
### *Real-time polymerase chain reaction*

RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 µg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. MiR-329 level was quantified by the mirVana qRT-PCR miRNA detection kit (Ambion, Austin, USA) in conjunction with real-time PCR with SYBR Green. After circle reaction, the threshold cycle (Ct) was determined and relative miR-329 level was calculated based on the Ct values and normalized to U6 level in each sample. PCR with the following primers: miR-329, Forward 5'-GGAAACACACCTGGTTAAC-3' and Reverse 5'-CATGCGTGTCTGGAGT-3'; U6, Forward 5'-CTCGCTTTGGCAGCACA-3' and Reverse 5'-AACGCTTACGAATTTGCGT-3'.

### *Immunohistochemistry*

The tissues were embedded in paraffin and cut into 5-µm sections. The sections showing typi-

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**Figure 1.** miR-329 expression in endometrial tissues was measured by qRT-PCR (A). mRNA (B) and protein (C) expression of CD146 in endometrial tissues were measured by qRT-PCR and western blotting respectively. Linear correlation plot of miR-329 and CD146 mRNA expression with endometrial thickness in infertility patients (D).

cal endometrial structures by hematoxylin and eosin (H&E) staining were included in this study. Immunohistochemistry was performed according to the SP kit instructions (Bioss Biotechnology, Beijing, China).

### Western blotting

Tissues and ESCs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 30  $\mu$ g of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with primary antibodies (Santa Cruz Biotechnology, CA, USA). After three washes with TBST, the membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with chemiluminescence (Thermo, USA).

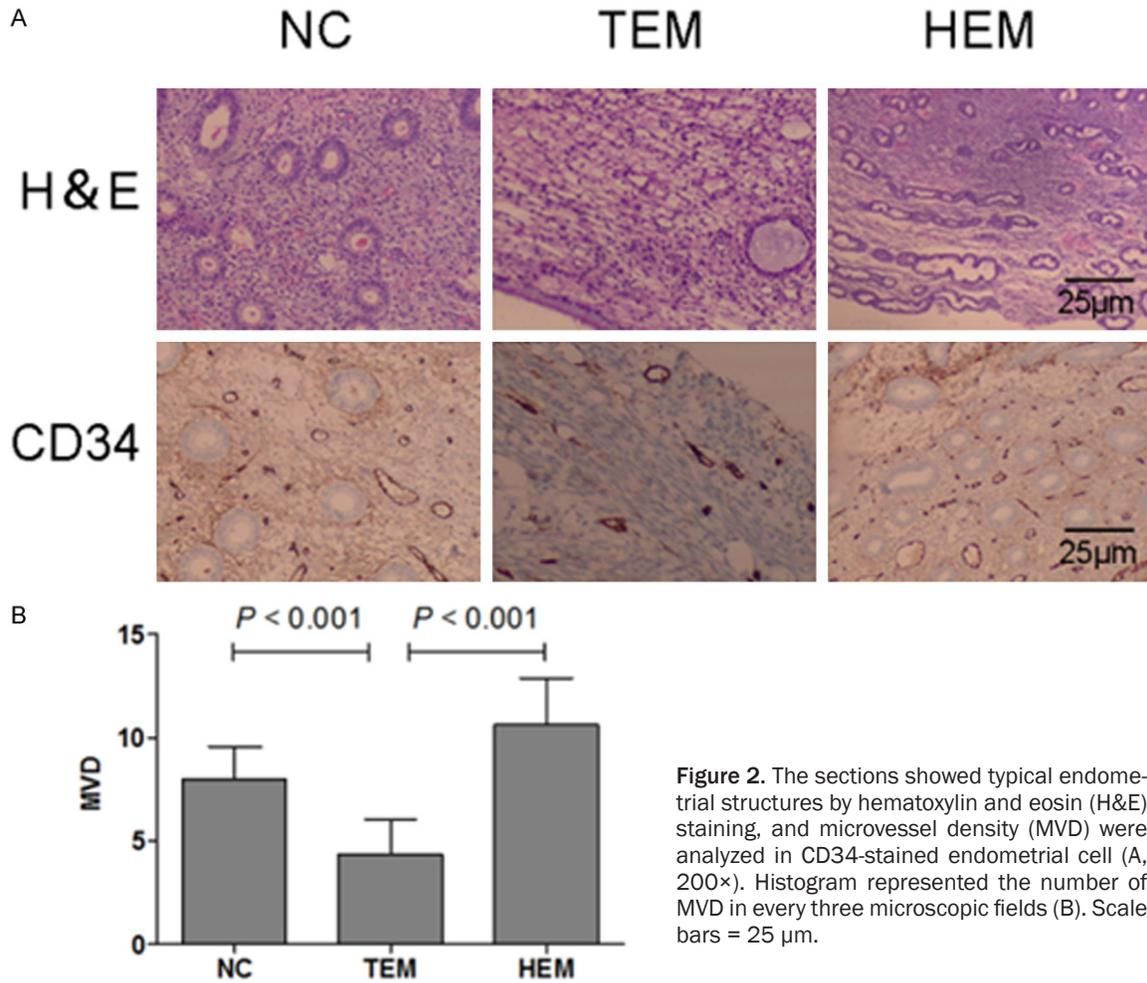
### Statistical analysis

The data from these experiments were reported as mean  $\pm$  standard deviation (SD) for each group. All statistical analyses were performed by using PRISM version 5.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA. Differences with  $P$  value of  $< 0.05$  were considered statistically significant.

## Results

### Correlation of miR-329 and CD146 with endometrial endometrial thickness

Earlier reports show that CD146 is extensively involved in the endometrial dysfunction, including hyperplasia and polyp. MiR-329 is considered as the best characterized miRNA associated with CD146 [10]. Moreover, miRNA target site prediction of CD146 was performed by online bioinformatics software: TargetScan, miRanda and mirBase. To explore the relationship between miR-329 and CD146, miR-329 and CD146 expression were examined by qRT-PCR in endometrial tissues, we found that miR-329 expression was significantly elevated in infertility patients with thinning endometrium compared with normal control, in contrast, miR-329 expression was significantly reduced in infertility patients with endometrial hyperplasia as compared to normal control (**Figure 1A**). Next, CD146 mRNA and protein expression were measured. As shown in **Figure 1B** and **1C**, CD146 mRNA and protein expression were increased in PEM group and decreased in TEM group as compared to that of patients in NC group. To test whether there was a relationship of miR-329 and CD146 expression with endometrial thickness in infertility patients, miR-329, CD146 and endometrial thickness were measured in the same individuals. As shown in



**Figure 2.** The sections showed typical endometrial structures by hematoxylin and eosin (H&E) staining, and microvessel density (MVD) were analyzed in CD34-stained endometrial cell (A, 200×). Histogram represented the number of MVD in every three microscopic fields (B). Scale bars = 25 μm.

**Figure 1D**, there was negatively correlation with the level of miR-329 and endometrial thickness in infertility patients ( $r = -0.876, P < 0.001$ ). However, there was positive correlation with the level of CD146 and endometrial thickness in infertility patients ( $r = -0.709, P < 0.001$ ).

#### Endometrial histomorphology

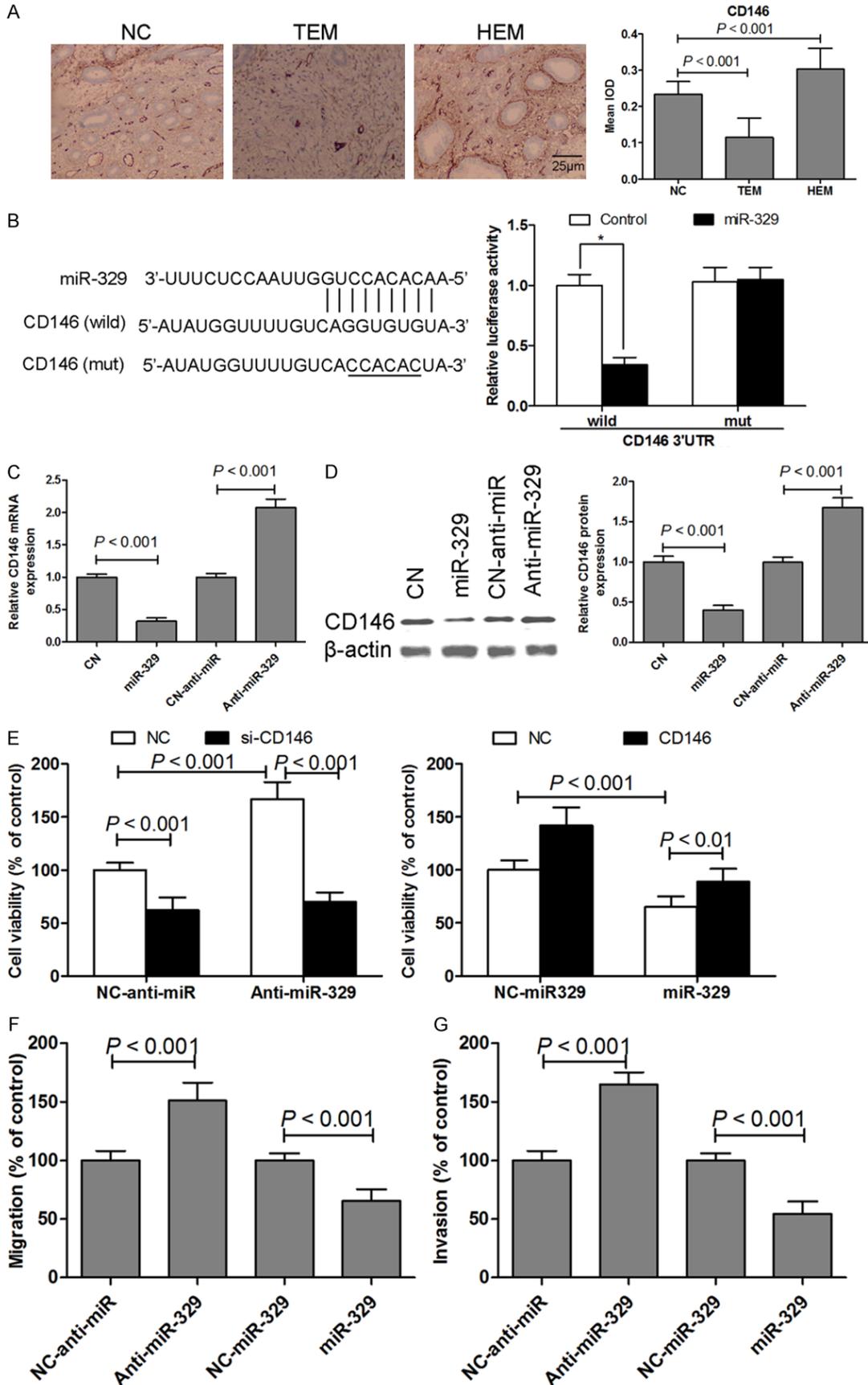
Histological analysis on endometrium in infertility patients was performed by H&E staining (**Figure 2A**). Simple columnar epithelium with very regular line-up of nuclei was discovered in the normal thickness of endometrium. Increased or decreased in the thickness of endometrium could induce structural disorder of endometrium, such as changing of the amount and size of glands and intercellular substance. Endometrial hyperplasia or normal endometrium showed a high MVD compared with TEM group; however, no differences were seen in

MVD between NC group and PEM group (**Figure 2A and 2B**).

#### MiR-329 targets CD146 in endometrium

CD146 is mainly expressed in the cytoplasm of the stromal endothelial cells. We first analyzed the levels of ESCs marker CD146 in the endometrial tissue from normal control and thinning/hypertrophic endometrium from infertility patients (**Figure 3A**). The immunohistochemical examination indicated that CD146 was strongly expressed in the endometrium during the proliferation phase in infertility patients; however, it was weakly expressed in infertility patients with thinning endometrium as compared to the normal development of endometrium. To determine whether miR-329 takes effect through direct binding to the 3'-UTR of CD146, we evaluated the alignment predicted by the TargetScan algorithm and found the potential miR-329

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**Figure 3.** CD146 immunohistochemical staining (A), schematic representation of the putative miR-329 binding site in the CD146 3'UTR as in Targetscan and luciferase activity assay (B), mRNA and protein expression of CD146 in the present of miR-329 loss-of function or gain-of function (C and D), cell viability was examined by CCK8 assay (E), migration and invasiveness were assessed using a migration and invasion assay (F and G). Data represent the mean  $\pm$  SD of three independent experiments. Scale bars = 25  $\mu$ m.

binding sites within the 3'-UTR of CD146 in human sapiens (**Figure 3B**). To further determine whether miR-329 regulated CD146 through the predicted binding sites in its 3'-UTR (**Figure 3B**), we utilized a construct carrying a luciferase gene upstream of the wild-type CD146 3'-UTR or the mutant 3'-UTR (containing mismatches in the seed region of the binding site). Cotransfection of HEK293 with this pMIR-REPORT construct containing mutant 3'-UTR of CD146, and reporter activity did not show much difference as compared to control group. Cotransfection with this luciferase construct containing wild-type CD146 3'-UTR and PLEmiR-329 resulted in a lower luciferase activity than control group, leading to a nearly 70% decline in the luciferase activity compared with control group (**Figure 3B**). The RT-PCR and western blotting results showed that the expression of CD146 was decreased with over-expressed miR329 in ESCs (**Figure 3C** and **3D**). However, inhibition of miR-329 in ESCs resulted in increased expression of CD146 at both the mRNA and protein levels (**Figure 3C** and **3D**). These results demonstrated that endogenous miR-329 indeed participated in the regulation of endometrial CD146. Furthermore, cell viability and motility were measured. We found that microRNA-329 via targeting CD146 regulated the cell viability and motility in ESCs. Down-regulation of miR-329 in ESCs increased cell proliferation, migration and invasion, whereas CD146 loss-of function suppressed cell proliferation, migration and invasion (**Figure 3E-G**). In contrast, up-regulation of miR-329 in ESCs decreased cell proliferation, migration and invasion, whereas CD146 gain-of function promoted cell proliferation, migration and invasion (**Figure 3E-G**).

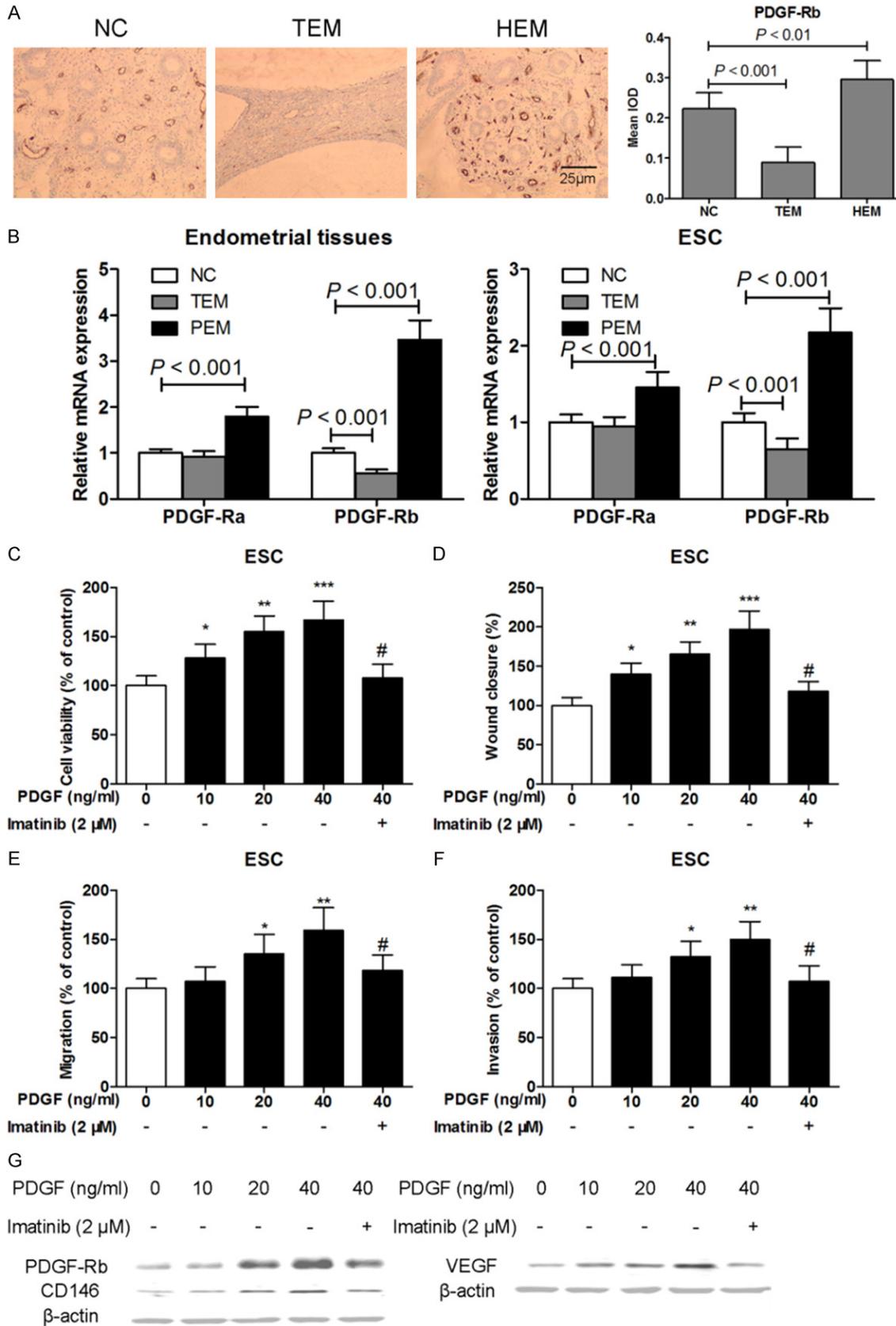
### *PDGF-Rb mediates endometrial dysfunction*

PDGF-Rb was mainly expressed in the cytoplasm of the stromal endothelial cells. PDGF-Rb was strongly expressed in the endometrium during the proliferation phase in infertility patients; however, it was weakly expressed in infertility patients with thinning endometrium

(**Figure 4A**). Moreover, both ESCs and endothelial tissues expressed the PDGF receptors PDGF-Ra and PDGF-Rb, and the level of PDGF-Rb was significantly higher than PDGF-Ra in ESCs and endothelial tissues. Importantly, PDGF-Ra and PDGF-Rb were significantly upregulated in PEM group as compared to NC group and TEM group (**Figure 4B**). To determine the role of PDGF-Rb in endometrial dysfunction, we analyzed PDGF function in ESCs. First, PDGF increased cell viability in a dose-dependent manner; however, cell viability was significantly inhibited in the present of imatinib, which is a PDGF-Rb antagonist (**Figure 4C**). The wound-healing and the in vitro migration and invasion assays indicated that PDGF also affected cell motility (**Figure 4D-F**). The ESCs exposure to imatinib, the migration and invasion were inhibited. Furthermore, PDGF strongly stimulated the expression of PDGF-Rb, CD146 and VEGF, imatinib treatment could reverse the expression of PDGF-Rb, CD146 and VEGF in ESCs (**Figure 4G**).

### **Discussion**

CD146, also known as MUC18, is a membrane glycoprotein that has suggested the perivascular location in many tissues. Recently, it has been identified as a novel marker of human ESCs [11]. Moreover, CD146 has been known to play a critical role in angiogenesis and induce ESCs differentiation [10, 11]. However, the mechanism of how CD146 expression is regulated during endometrial hyperplasia is still poorly understood. In the present study, we found that miR-329 as a negative regulator of endometrial CD146 could suppress CD146-mediated endometrial cell motility. In our study, we made several important observations. First, increased CD146 and decreased miR-329 were observed in proliferative phase of endometrium from infertility patients. Moreover, we found a highly negative correlation between miR-329 and CD146 (data not shown). Next, miR-329 loss-of-function increased and miR-329 gain-of-function decreased the expression of CD146 in ESCs. It was reasonable that miR-



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**Figure 4.** PDGF-Rb immunohistochemical staining (A), PDGF-Ra and PDGF-Rb mRNA expression were measured by qRT-PCR in endometrial tissues and ESCs (B), cell viability was examined by CCK8 assay in the present of PDGF or imatinib (C), cell motility was assessed using a wound-healing assay in the present of PDGF or imatinib (D), migration and invasiveness were assessed using a migration and invasion assay in the present of PDGF or imatinib (E and F), the protein expression of PDGF-Rb, CD146 and VEGF was measured by western blotting in the present of PDGF or imatinib (G). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control group; # $P < 0.05$  versus PDGF single treatment (40 ng/ml). Data represent the mean  $\pm$  SD of three independent experiments. Scale bars = 25  $\mu$ m.

329 regulated a distinct underlying molecular and pathogenic mechanism of endometrial hyperplasia by forming a negative feedback loop with CD146. Furthermore, PDGF strongly stimulated the expression of PDGF-Rb, simultaneously, PDGF could accelerate cell proliferation, migration, invasion via targeting PDGF-Rb. Together, and these observations suggested that miR-329 via targeting CD146 and PDGF-Rb played an important role in the pathological courses of endometrial hyperplasia.

In medicated frozen-thawed embryo replacement cycles, the lowest pregnancy rates were associated with endometrial thickness  $< 7$  mm and  $>14$  mm [3]. In this work, miR-329 via targeting CD146 might be a potential therapeutic target against endometrial hyperplasia, which could reduce the receptivity of endometrium and pregnancy rate. The expression of CD146 is decreased in the endometrial polyps (EP) patients as compared to that in the normal endometrial tissue at the proliferation phase. This result suggests that the endometrial stem cells were reduced in the EP patients [12]. A large body of evidence indicates that the activity of CD146 is significantly increased in a variety of tumors, and CD146 expression correlates with lymph node metastasis and is associated with poor 5-year overall survival [4]. In the present study, we found that there was positive correlation with the level of CD146 and endometrial thickness in infertility patients ( $r = -0.709$ ,  $P < 0.001$ ). More research into the causes of endometrial hyperplasia, we focused our efforts on miR-329 because it was the most significantly down-regulated miRNA, which was confirmed by microarray analysis. Moreover, miR-329 directly downregulated CD146 levels through direct binding the 3'-UTR of CD146. These results demonstrated that miR-329 via targeting CD146 associated with endometrial dysfunction and might be a potential therapeutic target against endometrial hyperplasia.

PDGF is considered one of the major regulators of endometrial cell biology [13, 14]. Indeed, dis-

tinct PDGF isoforms, especially PDGF-BB, have been shown to stimulate endometrial cell proliferation, invasiveness and migration [15]. Moreover, PDGF can induce endometrial cell proliferation and motility [13]. Consistent with this report, we results indicated that PDGF increased cell viability in a dose-dependent manner and affected cell motility. Intriguingly, we found that in response to PDGF the expression of PDGF-Rb, CD146 and VEGF was significantly upregulated in ESCs, and imatinib inhibited PDGF-induced endometrial cell motility and the target protein expression. These results suggested that PDGF could accelerate cell proliferation, migration, invasion via targeting PDGF-Rb; PDGF-Rb could play an important role in endometrial dysfunction.

In conclusion, our results provide novel insights into the underlying mechanisms of CD146 and PDGF-Rb in endometrial dysfunction. Intriguingly, miR-329 may play a potential therapeutic avenue for the treatment of endometrial hyperplasia in infertility patients.

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### Disclosure of conflict of interest

None.

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