Original Article

MiR-199a inhibits the angiogenic potential of endometrial stromal cells under hypoxia by targeting HIF-1α/VEGF pathway

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Abstract: We previously reported that miR-199a suppressed the invasiveness of endometrial stromal cells (ESCs) by targeting IkappaB kinase beta (IKKβ). This study was to investigate the role of miR-199a in the angiogenic potential of ESCs under hypoxia. Forced overexpression of miR-199a in ESCs significantly attenuated its angiogenic potential under hypoxia. Moreover, miR-199a down-regulated the expression level of vascular endothelial growth factor-A (VEGF-A) in ESCs under hypoxic conditions. To delineate the mechanism by which miR-199a reduced VEGF-A production, further analysis of the target genes of miR-199a showed that miR-199a targeted both VEGF-A and Hypoxia-inducible factor (HIF)-1α in ESCs. Our findings indicate that miR-199a may attenuate the angiogenic potential of ESCs under hypoxia partly through HIF-1α/VEGF-A pathway suppression. Therefore, miR-199a may play pivotal roles in the pathogenesis of endometriosis and may become a potential therapeutic target of this disease.

Keywords: Endometriosis, miR-199a, angiogenesis, VEGF-A, HIF-1α

Introduction

Endometriosis, a common and estrogen-dependent disease, is characterized by the growth of endometrial tissue outside the uterus. Currently, the most widely accepted hypothesis of endometriosis development is retrograde menstruation and implantation theory, which suggests that endometrial tissue may flow back through fallopian tubes at menstruation, implant within the peritoneal cavity and become endometriosis [1]. To implant in ectopic sites, the endometriotic lesion need to grow its own blood supply by generating new blood vessels from pre-existing ones, the essential process called angiogenesis [2, 3].

Angiogenesis, a complex process involving endothelial cell proliferation, migration, invasion and tube formation, is regulated by local proangiogenic cytokines and inhibitors [4, 5]. Among numerous alterations of proangiogenic factors in the angiogenesis of ectopic endometrium, vascular endothelial growth factor A (VEGF-A) is thought to be a pivotal mediator in the pathogenesis [3]. VEGF-A, a member of VEGF family, includes three major isoforms, designated as VEGF165, VEGF189 and VEGF121 [6]. VEGF165 is the most abundant and active one and was reported to be over-expressed in the peritoneal fluid of endometriosis patients [7, 8]. Besides, VEGF-A level was higher in the eutopic endometrium of women with endometriosis than that of women without the disease [9, 10]. Since it is well known that VEGF-A can stimulate angiogenesis and increase vessel permeability, endometrium of women with endometriosis may have a higher angiogenic potential and may be more likely to implant in ectopic sites than that of women without the disease. Moreover, it is reported that anti-VEGF antibody treatment significantly inhibits the endometriotic lesion development in a mouse model of surgically induced endometriosis [11]. Thus, VEGF-A is crucial for the establishment of endometriotic lesions.
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VEGF-A production could be regulated by various stimuli, among which hypoxia is the most potent one [12]. Under hypoxic environments, HIF-1α translocates into the nucleus where it heterodimerizes with HIF-1β and binds to hypoxia-responsive elements on various genes, including VEGF-A [13]. It is known displaced endometrium may initially lack blood supply and, thus, suffer from ischemia and hypoxia, leading to the activation of HIF-1α/VEGF pathway, a key player in angiogenesis and endometriotic lesion formation [14, 15]. However, the molecular mechanism by which the HIF-1α/VEGF pathway is regulated during the early development of endometriosis remains largely unexplained.

MiRNAs are small non-coding RNAs of 20-22 nucleotides, which post-transcriptionally regulate gene expression and can control a broad spectrum of normal and pathological cellular functions [16-18]. First discovered in 2003 [19, 20], miR-199a has been shown to play important roles in the regulation of the angiogenic process [21-23]. For example, miR-199a inhibition promotes wound angiogenesis by regulating Ets-1/MMP-1 pathway [21]. Moreover, miR-199a overexpression inhibited tumor-induced angiogenesis by decreasing HIF-1α and VEGF expression [23]. Recently, our group found that the miR-199a expression level is lower in the eutopic endometrium of women with endometriosis than that of women without the disease [24]. Moreover, miR-199a over-expression attenuated endometrial stromal cell (ESC) invasiveness through suppressing IkappaB kinase beta (IκB)/nuclear factor-kappa B (NF-κB) pathway [24]. However, the role of miR-199a in the endometriosis-associated angiogenesis under hypoxia remains largely unknown.

In the current study, we postulate that miR-199a could play a role in the endometriotic lesion formation by regulating the angiogenic potential of endometrial cells under hypoxia. Therefore, the angiogenic potential of miR-199a transfected ESCs under hypoxia was tested. Moreover, we found that HIF-1α/VEGF pathway, which is regulated by miR-199a, may contribute to the angiogenic potential of ESCs under hypoxia. These findings further reveal the relevance of miR-199a in the pathogenesis of endometriosis.

Materials and methods

Tissue collection, cell culture and transfection

Eutopic endometrial tissues were obtained from endometriosis patients undergoing laparoscopy (n=8; age range: 26 to 36 years). Isolation and primary culture of ESCs was carried out as previously described [25, 26]. All experimental procedures were approved by the ethical committees of Renji hospital affiliated to Shanghai Jiao Tong University, China. The written informed consents were given to all the patients. MiRNAs were synthesized and transfected into ESCs as described in our previous work [24]. The human umbilical vein cell line, EA.hy926, was ordered from ATCC (Manassas, VA, US) and cultured in DMEM complete media. Unless otherwise indicated, EA.hy926 was serum-starved overnight prior to being used for the proliferation, migration, invasion and tube formation assay.

Cell proliferation assay

Endothelial cells (EA.hy926) were seeded in three to four replicates into 96-well plates. The conditioned media (CM) were prepared from miR-199a or NC transfected ESCs cultured under normoxia or hypoxia (1% O2) in serum-free media for 24 h. After treatment with the indicated CM for 72 h, endothelial cell viability was assessed using Cell Counting Kit-8 (Dojindo, Kami Mashiki-Gun, Kumamoto, Japan).

Cell migration assay

The Boyden chamber (8-µm pore size, Millipore, USA) was used to measure the migratory ability of EA.hy926 cells. Cells, re-suspended in the indicated CM, were plated in the top chamber. The medium containing 20% fetal bovine serum was used as a chemoattractant in the bottom chamber. After incubation for 8 h, the invading cells on the lower side of the membrane were fixed, stained and counted.

Matrigel invasion assay

The Matrigel pre-coated Boyden chamber was used to measure the invasive ability of EA.hy926 cells. Cells, re-suspended in the indicated CM, were plated in the top chamber. The medium containing 20% fetal bovine serum
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**Figure 1.** MiR-199a attenuates the angiogenic potential of ESCs in vitro. A. Cell proliferation assay. Serum-starved EA.hy926 cells were stimulated with CM. After 72 h, CCK-8 assay was used to determine the number of cells. B. Cell migration assay. CM stimulated cells, which had migrated for 8 h, were counted using a microscope (200× magnification). C. Cell invasion assay. CM stimulated cells, which had invaded for 24 h, were counted. D. Tube formation assay. EA.hy926 cells suspended in CM were plated on growth factor-reduced Matrigel to form capillary tubes. After
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was used as a chemoattractant. After incubation for 24 h, the invading cells were fixed, stained and counted.

**Tube formation assay**

**EA.hy926 cells**, counted and re-suspended in CM, were seeded on the Matrigel pre-coated 96-well plates at density of 4×10^4 cells/well. After incubation for 12 h, tube formation was observed, photographed and quantified.

**Quantitative real-time RT-PCR (qRT-PCR) analysis**

Total RNA was isolated by suitable reagents. SYBR Green qRT-PCR was used to detect VEGF-A, HIF-1α and GAPDH. 2^{ΔΔCt} method was used to calculate the relative gene expression levels. Primers used were as follows: for VEGF-A, 5'-CTTGCCGGTGCTCTTACC-3' (forward) and 5'-CACACAGATGGCTTGAAAG-3' (reverse); for HIF-1α, 5'-GCAAGCCCTGAAAGCG-3' (forward) and 5'-GGCTGTCCGACTTTGAG-3' (reverse); for GAPDH, 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGTCTAG-3' (reverse).

**ELISA assay**

CM was collected, centrifuged and stored at -80°C. The VEGF-A secretion level was determined by a commercially available ELISA kit (R&D Systems, Canada).

**Western blot analysis**

Protein extracts for western blot were prepared using RIPA buffer at 48 h post transfection. The antibodies used were anti-HIF-1α (CST, Danvers, MA, USA) and anti-GAPDH (Santa Cruz, CA, USA). Western blotting was performed as previously described [27].

**Reporter gene assay**

The sequence containing the predicted miR-199a binding site within VEGF-A 3’-UTR TGTTATTGGGTCTTACG or a mutant sequence TGTTATTGGCTTGGCATG was cloned into the luciferase reporter vector, generating Luc. VEGFA and Luc.control1 vectors, respectively. Similarly, the sequence containing the predicted miR-199a binding site within the HIF1A 3’-UTR TTTTTGACACTTGTTGAGCATTAC or a mutant sequence TTTTTGACACTTGCTCATTAC were cloned into the vector, generating Luc.HIF1A and Luc.control2 vectors, respectively. Reporter Gene Assay was performed as previously described [24].

**Statistical analysis**

Data, presented as means ± SD, was analyzed by SPSS software using t-test or ANOVA. P<0.05 was defined as statistically significant.

**Results**

**MiR-199a attenuates the angiogenic potential of ESCs under hypoxia**

Angiogenesis is the essential process for endometriotic lesions formation [3, 28]. We evaluated the angiogenic ability of NC and miR-199a overexpressing ESCs under normoxia and hypoxia through using endothelial cell (EA.hy926) proliferation, migration, invasion and tube formation assays. We found that the conditioned medium (CM) prepared from the ESCs cultured under hypoxia significantly promoted the proliferation (**Figure 1A**), migration (**Figure 1B**), invasion (**Figure 1C**) and tube formation ability (**Figure 1D**) of endothelial cells. However, transfection of ESCs cells with miR-199a profoundly suppressed these effects (**Figure 1A-D**). Thus, miR-199a may inhibit the angiogenic potential of ESCs under hypoxic conditions.

**MiR-199a suppresses the VEGF-A secretion from ESCs**

VEGF-A, a key pro-angiogenic factor in endometriosis-associated angiogenesis [29], could be induced under hypoxia [12]. To explore whether miR-199a could affect the VEGF-A secretion of ESCs under hypoxia, we measured the mRNA and the protein secretion levels of VEGF-A in miR-199a transfected cells cultured under normoxia or hypoxic conditions. Hypoxia treatment
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resulted in significant increases of VEGF-A mRNA and protein levels as measured by Quantitative RT-PCR analysis and ELISA (Figure 2A, 2B). Markedly, miR-199a transfection profoundly inhibited the hypoxia-induced VEGF-A transcription and secretion (Figure 2A, 2B). Moreover, miR-199a also inhibited the VEGF secretion level of ESCs under normoxia (Figure 2B).

MiR-199a targets VEGF-A in ESCs

Figure 3A shows the 3’UTR of VEGF-A contains a potential target of miR-199a. ESCs were co-
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transfected with a luciferase reporter containing either the miR-199a target region or a mutant, in addition to NC or miR-199a. As expected, miR-199a overexpression effectively inhibited the luciferase activity of Luc.VEGFA, while NC miRNA did not have that effect. Besides, both NC and miR-199a miRNA did not affect the luciferase activity of Luc.control1 (Figure 3B). These results suggest that the inserted fragment of VEGF-A could be targeted by miR-199a. We then transfected miR-199a into ESCs and tested the VEGF-A expression level. MiR-199a overexpression reduced VEGF-A secretion, but did not affect the VEGF-A mRNA level (Figure 3C, 3D).

MiR-199a targets HIF1A in ESCs

HIF1A was confirmed as a miR-199a target in cardiac myocytes [22]. Figure 4A shows the 3'UTR of HIF1A contains a potential target of miR-199a. To determine whether miR-199a also targets HIF1a in ESCs, ESCs were co-transfected with a luciferase reporter containing either the miR-199a target region or a mutant, in addition to NC or miR-199a. As expected, miR-199a overexpression effectively inhibited the luciferase activity of Luc.HIF1A, while NC miRNA did not have that effect. Besides, both NC and miR-199a miRNA did not affect the luciferase activity of Luc.control2 (Figure 4B). These results suggest that the inserted fragment of HIF1A could be targeted by miR-199a. We then transfected miR-199a into ESCs and tested the HIF1A expression level. MiR-199a overexpression reduced HIF1A expression under hypoxia, but did not affect the HIF1A mRNA level (Figure 4C, 4D).

Discussion

MiR-199a, first characterized in 2003 [19, 20], has been shown to be associated with many different types of human disease [30-32], but the information on its significance in angiogenesis, especially endometriosis-associated angiogenesis, is scanty. By elucidating the contribution of miR-199a to the endometrial angiogenesis, the present study now provides the first evidence demonstrating that endogenous miR-199a blocks the angiogenic potential of ESCs under hypoxia by targeting HIF-1α/VEGF pathway.
In spite of the relatively high prevalence of endometriosis in women all over the world, the mechanisms of this debilitating disease remain largely unknown. Currently, the most widely accepted model of endometriotic lesion formation is that endometrial tissue flows back through fallopian tubes and implants in ectopic sites through steps of adhesion, invasion, angiogenesis and proliferation [33, 34]. During this process of transplantation, angiogenesis, which acquires new blood vessels from surrounding vasculatures, is the most basic requirement for endometriotic lesion formation [35, 36]. Besides, according to the theory of retrograde menstruation and transplantation, we can speculate that displaced endometrial tissue is initially under hypoxic conditions, a strong stimulus for angiogenesis [37], until it makes a connection with the systemic circulation through new blood vessel formation. Thus, hypoxia may be an early phenomenon in endometrial tissue transplantation and a crucial factor for endometrial angiogenesis. Our group has recently reported that miR-199a expression level was lower in the eutopic endometrium of women with endometriosis than that of women without the disease [24], which suggest that miR-199a may play a role in the initiation of endometriosis. As miR-199a has been shown to be involved in both wound angiogenesis [21] and tumor angiogenesis [23], we examined whether miR-199a over-expression would impact the angiogenic potential of ESCs under hypoxia. Our results showed that the conditioned media (CM) prepared from miR-199a over-expressing ESCs cultured under hypoxia could inhibit endothelial cell (EA hy926) proliferation, migration, invasion and tube formation. Our results strongly suggest that miR-199a may play a crucial role in endometrial angiogenesis under hypoxia. Chan et al. reported that miR-199a in endothelial cells negatively regulated angiogenesis in vivo and in vitro by targeting Ets-1 [21]. Different from their study, which focused on autonomous angiogenesis of endothelial cells, our study concentrates on ESCs and endometriosis-associated angiogenesis. Besides, He et al. reported that overexpression of miR-199a down-regulated tumor-associated angiogenesis by decreasing the expression levels of HIF-1α and VEGF protein [23]. Different from their study, which focused on the angiogenic potential of cancer cells under normoxia, our study concentrates on the endometrial angiogenesis under hypoxia. Although miR-199a seems to function as an anti-angiogenic factor under most circumstances, Pencheva et al. reported that miR-199a could work as a pro-angiogenic factor in melanoma that promotes angiogenesis by targeting apolipoprotein E (ApoE) [38]. Together, the results of these studies suggest that miR-199a may have different regulatory mechanisms of angiogenesis in different cell types and under different conditions.

VEGF, the most prominent angiogenic growth factor in endometriosis-associated angiogenesis [3, 29], could be induced by hypoxia [12]. Sharkey et al. reported that endometrial cells cultured under hypoxia secreted higher level of VEGF than that cultured under normoxia [39]. Moreover, hypoxia could increase the VEGF expression level in a mouse model of surgically induced endometriosis [14]. Thus, we next examined whether miR-199a inhibited the angiogenic potential of ESCs under hypoxia through regulating the VEGF-A secretion level of the cell. We confirmed that VEGF-A mRNA expression level was down-regulated significantly in miR-199a transfected ESCs cultured under hypoxia. Moreover, VEGF-A protein secretion level was decreased significantly in both hypoxia and normoxia group. These results suggest that miR-199a may attenuate the angiogenic potential of ESCs under hypoxic conditions partly through reduced VEGF-A secretion. Interestingly, miR-199a inhibits the VEGF-A secretion, however, it did not affect the angiogenic potential of ESCs under normoxia. This may be partly due to the relatively lower VEGF-A secretion level of ESCs under normoxia but the exact mechanism needs further research.

It is reported that miR-199a directly targets VEGF-A in endometrial mesenchymal stem cells [40]. However, certain miRNA may target different genes in different cell types [41, 42]. For instance, miR-199a does not target HIF-1α in ovarian cancer cells [23] although it has been established to target HIF-1α in cardiac myocytes [22]. Thus, we use dual luciferase reporter gene assays to determine whether miR-199a also targets VEGF-A in ESCs. The results showed that miR-199a could bind to the seed region of the 3′UTR of VEGF-A mRNA, which confirmed that VEGF-A is a functional target of miR-199a in ESCs. Besides, miRNA can induce
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RNA degradation or translational arrest of the target gene [43]. Our results showed that miR-199a inhibited VEGF-A secretion but did not affect the mRNA level of VEGF-A, which suggests miR-199a played a translation-inhibitory role. Interestingly, after miR-199a transfection, VEGF-A secretion level was attenuated to a lesser degree under normoxia than that observed under hypoxia. Moreover, the mRNA level of VEGF-A was almost unchanged in miR-199a transfected ESCs under normoxia, while it was down-regulated significantly under hypoxia. It is thus suggested that other functional targets of miR-199a could contribute to the reduced VEGF-A expression level under hypoxia.

HIF-1α, a hypoxia responsive transcription factor, could promote VEGF-A secretion strongly by binding to hypoxia-responsive elements on the VEGF-A gene [37]. Besides, HIF-1α has been validated as a direct target of miR-199a in cardiac myocytes [22]. Because miRNA targets may be tissue specific [41, 42], we use dual luciferase reporter gene assays to determine whether miR-199a also targets HIF-1α in ESCs. The results showed that miR-199a could bind to the seed region of the 3’UTR of HIF-1α mRNA, which confirmed that HIF-1α is a functional target of miR-199a in ESCs. Besides, miR-199a inhibited the protein level of HIF-1α but almost did not affect the mRNA level under hypoxia, which suggests miR-199a played a translation-inhibitory role. These findings are consistent with those of Song et al.’s research, which suggested that miR-199a inhibits target protein synthesis through translational arrest [44]. However, they are different from Shen et al.’s research data, which suggest that miR-199a regulate the target protein synthesis through RNA degradation [45]. Together, our results showed that miR-199a negatively regulated angiogenesis by targeting two essential genes, VEGF-A and HIF1A, in the HIF-1α/VEGF signaling pathway in ESCs.

In conclusion, we have identified that miR-199a could inhibit the angiogenic potential of ESCs under hypoxia partly through targeting HIF-1α/VEGF-A signaling pathway (Figure 5). The results of the current study, together with the findings from our previous report [24], indicate that miR-199a could not only inhibit ESC invasiveness but also exert potent anti-angiogenic effect on endometriosis-associated angiogenesis, which suggests miR-199a could become a potential therapeutic target of endometriosis. Nevertheless, further in vivo studies of miR-199a are deemed necessary in the future in order to develop miR-199a based treatment of endometriosis.

Figure 5. A dual negative regulation model of HIF-1α/VEGF-A pathway by miR-199a. Under hypoxia conditions, HIF-1α accumulation in ESCs leads to increased VEGF-A production, which promotes the proliferation, migration, invasion and tube formation of endothelial cells. However, when miR-199a was transfected into ESCs, it could down-regulate its target genes, including HIF-1α and VEGF-A, thereby suppressing the positive signaling and attenuating the angiogenic potential of ESCs.

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Disclosure of conflict of interest

None.

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References

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