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Hypoxia responsive miR-210 promotes cell survival and autophagy of endometriotic cells in hypoxia

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Abstract. – OBJECTIVE: Hypoxia may play a role in the survival of ectopic endometrial cells. This study aimed to explore how hypoxia responsive miR-210 is involved in cell survival and autophagic response of endometriotic cells.

MATERIALS AND METHODS: The expression of hypoxia-inducible factor 1-alpha (HIF-1α) and miR-210 in eutopic and ectopic endometrial tissues were measured. The expression changes of HIF-1α and miR-210 in ovarian endometriotic cell line CRL-7566 after hypoxic culture were further explored. The influence of miR-210 on cell viability and apoptosis was quantified using CCK-8 assay and flow cytometry analysis. The effect of miR-210 on Bcl-2 expression and the effect of miR-210/Bcl-2 axis on autophagy in the cells were measured by Western blot analysis.

RESULTS: Ectopic lesion had stronger HIF-1α positive signals, as well as more HIF-1α positive cells per visual field than the eutopic endometrium. MiR-210 expression was also elevated in the ectopic lesions. In *in-vitro* models, CRL-7566 cells had significantly higher expression of HIF-1α and miR-210 after hypoxic treatment. MiR-210 overexpression partly preserved cell viability in hypoxia, while miR-210 knockdown facilitated the loss of cell viability. In addition, miR-210 significantly attenuated hypoxia-induced apoptosis in CRL-7566 cells. Enforced miR-210 overexpression significantly promoted autophagy in hypoxia. Knockdown of endogenous Bcl-2 significantly enhanced autophagy, the effect of which was similar to that of miR-210.

CONCLUSIONS: The hypoxia-induced higher miR-210 expression may contribute to pathological development of endometriosis at least through enhancing cell survival and promoting autophagy via Bcl2/Beclin-1 axis.

Key Words:
Hypoxia, miR-210, Autophagy, Endometriosis.

Introduction

Endometriosis is a common benign gynecological disease characterized as the migration and proliferation of endometrial cells outside of the uterus[1,2]. It affects about 10% to 15% of premenopausal women[3-5]. The pathogenesis of endometriosis is likely multifactorial[6-8]. Some hypotheses have been proposed to explain the migration, implantation and survival of ectopic endometrial tissue and stroma, such as retrograde menstrual reflux[9], ectopic presence of endometrial stem cells[10] and defects in immune system[11]. However, the mechanisms underlying the capacity of ectopic endometrial cells to evade the overall control of the apoptotic program has not been fully revealed.

There are emerging studies suggesting that hypoxia may play a role in the survival and angiogenesis of ectopic endometrial cells[8,12,13]. Since miRNAs regulate a broad spectrum of normal and pathological cellular functions, some recent works also reported that miRNAs may play pivotal roles in the pathogenesis of endometriosis in hypoxia[14,15]. For example, hypoxia-induced miR-20a increases ERK phosphorylation and expression of angiogenic genes in endometriotic stromal cells[16]. Enforced miR-199a expression in endometrial stromal cells significantly attenuated its angiogenic potential under hypoxia[17]. Hypoxia is a well-known inducer of autophagy[18]. The autophagic response may also contribute to the cell survival and lesion maintenance of endometriosis[19]. One recent article[19] observed that autophagy is upregulated in ovarian endometrio-

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sis. However, the association between hypoxia and autophagy in the pathogenesis of endometriosis is not quite clear.

MiR-210 is a hypoxia responsive miRNA in multiple types of cancer, such as colon cancer[20], prostate cancer[21] and renal cancer[22] and also contributes to pathogenesis of endometriosis through activation of signal transducer and activator of transcription 3[23]. In this study, we explored the association between hypoxia-inducible factor 1-alpha (HIF-1α) and miR-210 in eutopic and ectopic endometrial tissues and further studied the involvement of miR-210 in cell survival and autophagy of endometriotic cells in hypoxia.

Materials and Methods

Human Tissues Samples

This study was approved by the Ethics Committee of Binzhou Medical University and the patients of reproductive age (range 30-46 years) were recruited from the Hospital Affiliated to the University. Informed consent was obtained from the patients before tissue collection. Ectopic endometrial tissues were obtained from 10 patients with ovarian endometriosis who underwent primary surgical laparoscopy. Eutopic endometrial tissues were obtained from 10 sterile women with normal menstrual cycle whose infertility was due to tubal factor. Endometriosis was excluded by laparoscopy. All tissues samples were histologically confirmed by pathologists.

Immunohistochemical Staining

Tissues were prepared according to the methods introduced in one previous study[24]. The tissues sections were incubated with primary antibodies to HIF-1α (ab82832, 1:100, Abcam, Cambridge, MA, USA) at 4°C mins in a humidified chamber overnight. Then the slides were incubated with biotinylated anti-rabbit secondary antibody for 30 minutes and then washed with phosphate buffered saline (PBS) for 5 mins. After that, the slides were incubated in streptavidin-horseradish peroxidase (HRP) solution for another 30 minutes. The HRP activity was detected using DAB as substrate. Counterstaining was performed using Harris hematoxylin. Negative controls were performed by incubation in PBS without the presence of primary antibody. Then, the slides were examined under a transmission light microscope.

Cell Culture

Ovarian endometriotic cell line CRL-7566 was obtained from ATCC. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated FBS in an incubator with a humidified air and 5% CO_2 at 37°C. For hypoxia induction, the cells were exposed to hypoxic environment (2% O_2) in a modulator chamber for indicating duration.

Reagent and Cell Treatment

MiR-210 mimics, inhibitors (IH), Bcl-2 siRNA and the corresponding scrambled negative controls were all purchased from Ribobio (Shanghai, China). To overexpress or inhibit miR-210 expression in CRL-7566 cells, the cells were transfected with 100 nM miR-210 mimics or inhibitors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To knockdown of endogenous Bcl-2, the cells were transfected with 100 nM Bcl-2 siRNA using Lipofectamine 2000 (Invitrogen). 3-methyladenine (3-MA), an autophagy inhibitor was purchased from Sigma-Aldrich (St. Louis, MO, USA). To inhibit autophagy, CRL-7566 cells were treated with 3-MA (5 mM) 1 hour before hypoxia, for 24 hours.

QRT-PCR Analysis of HIF-1α and miR-210 Expression

Total RNA and total miRNAs from tissue or cell samples were extracted using Trizol reagent (Invitrogen) the miRVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction respectively. To quantify HIF-1α mRNA, complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). Then QRT-PCR was performed by using the gene specific primers (HIF-1α, forward, 5'-ACGAGAGGTTCCCTAATTTCCA-3', reverse, 5'-ATGCCACCAGTACATTGGGAT-3'; GAPDH, forward, 5'-GGGAAGCTTGTCATCAATGG-3', reverse, 5'-CATCGCCCCACTTGATTTTG-3') and SYBR Premix Ex Taq II (TaKaRa) in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). To quantify miR-210 expression, cDNA for miRNA was synthesized using the Taqman MicroRNA Reverse Transcription Kit (Ambion, Carlsbad, CA, USA). Mature miR-210 was detected using the TaqMan® microRNA assay for miR-210 (Ambion) and TaqMan® universal PCR master mix (Ap-

plied Biosystems) in an ABI 7500 Real-Time PCR system (Applied Biosystems). The relative amounts of mRNA and miRNA were calculated using the 2-ΔΔCt methods.

Western Blot Analysis of HIF-1α and Autophagy Related Proteins

Tissue or cell samples were firstly lysed using a lysis buffer (Beyotime, Shanghai, China). For the samples used for autophagy detection, proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA) was added. Then, the protein concentration was quantified using a BCA protein assay kit (Beyotime). The samples were separated on 10% SDS-PAGE gel and then transferred to PVDF membranes. The membranes were then blocked in 5% nonfat milk in TBST at room temperature for 1 hour and then incubated with anti-HIF-1α (1:1000, #3716, Cell Signaling, Danvers, MA, USA), anti-LC3B (1:3000, ab51520, Abcam), anti-active caspase-3 (1:1000, ab2302, Abcam) or anti-GAPDH (1:1000, Abcam, ab181602) overnight at 4°C. After three consecutive washing using TBST, the membranes were incubated with HRP conjugated secondary antibodies for another 1 hour at room temperature. Then the proteins were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA). The signal intensity was quantified using ImageQuant 5.2 (GE Healthcare, Piscataway, NJ, USA). The relative gray-scale value of HIF-1α versus GAPDH in normoxia group was set as 1.

Analysis of Cell Viability and Cell Apoptosis

Viability of the cells after indicating treatment was measured using the Cell Counting Kit-8 (CCK-8; CK04, Dojindo, Kumamoto, Japan) in combination with a spectrophotometer. The ratio of apoptotic cells with quantified using the Annexin V-FITC Apoptosis Detection Kit (V13241, Invitrogen) with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Data were reported as means ± standard deviation (SD). Comparison between groups was performed using Student's *t*-test with SPSS 18.0 software (SPSS, Chicago, IL, USA). p value of < 0.05 was considered as statistically significant.

Results

HIF-1α is Overexpressed in Ectopic Tissues and is Significantly Correlated with miR-210 Expression

Firstly, we detected the distribution and quantified the number of cells with positive HIF-1α protein by immunohistochemistry in eutopic endometrium and ectopic lesion of ovarian endometriosis. The results showed that the HIF-1α mainly distributed in the glandular component in eutopic endometrium (Figure 1A). However, in the ectopic lesions, HIF-1α positive staining was observed in both glandular component and stromal cells (Figure 1A). In addition, ectopic lesion had stronger HIF-1α positive signals, as well as more HIF-1α positive cells per visual field than eutopic endometrium (89 ± 9 *vs.* 24 ± 6) (Figure 1A-B). By performing qRT-PCR analysis based on tissues of ectopic lesions and eutopic endometrium, we also confirmed substantially increased HIF-1α mRNA in the ectopic lesions (Figure 1C). Previous studies reported that miR-210 is a hypoxia responsive miRNA[22] and might be involved in the pathological development of endometriosis[23]. Therefore, we decided to investigate its function in endometriosis. We confirmed that the expression of miR-210 was significantly higher in the ectopic lesions than in the eutopic endometrium (Figure 1D). In *in-vitro* models, the ovarian endometriotic cell line CRL-7566 had significantly higher expression of HIF-1α expression at both mRNA and protein level after hypoxic treatment (Figure 1D-E). Besides, miR-210 expression was also increased after hypoxia treatment (Figure 1F).

MiR-210 Increases Cell Viability and Reduces Cell Apoptosis of Endometriotic Cells in Hypoxia

Since miR-210 is a hypoxia responsive miRNA in endometriosis, we then investigated its functions in hypoxia. CRL-7566 cells were firstly transfected with miR-210 mimics (Figure 2A) or inhibitors (Figure 2C). By measuring cell viability of the cells with miR-210 overexpression or knockdown in hypoxia up to 48 hours, we observed that miR-210 overexpression partly preserved cell viability in hypoxia (Figure 2B), while miR-210 knockdown facilitated the loss of cell viability (Figure 2D). Spontaneous apoptosis is a natural cellular response under hypoxia[18]. Then, we studied the function of miR-210 in cell apoptosis. Flow cytometry analysis showed that

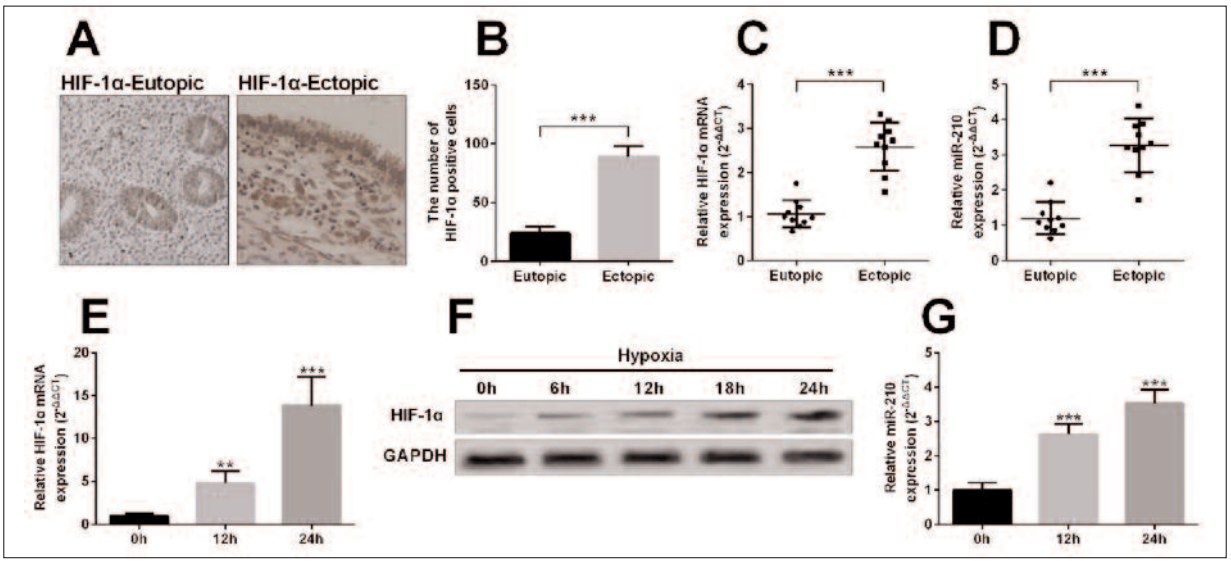

Figure 1. HIF-1α is overexpressed in ectopic tissues and is significantly correlated with miR-210 expression. ***A,*** Expression of HIF-1α was examined by immunohistochemical (IHC) staining in eutopic tissues of tubal infertility and ectopic tissues of patients with endometriosis. ***B,*** Quantification of the HIF-1α positive cells per visual field. ***C*** and ***D,*** QRT-PCR analysis of HIF-1α ***(C)*** and miR-210 expression ***(D)*** in eutopic and ectopic endometrial tissues. ***E*** and ***F,*** QRT-PCR ***(E)*** and Western blot ***(F)*** analysis of HIF-1α expression in CRL-7566 cells in hypoxic culture at indicating time points. ***G,*** QRT-PCR analysis of miR-210 expression in CRL-7566 cells in hypoxic culture at indicating time points. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

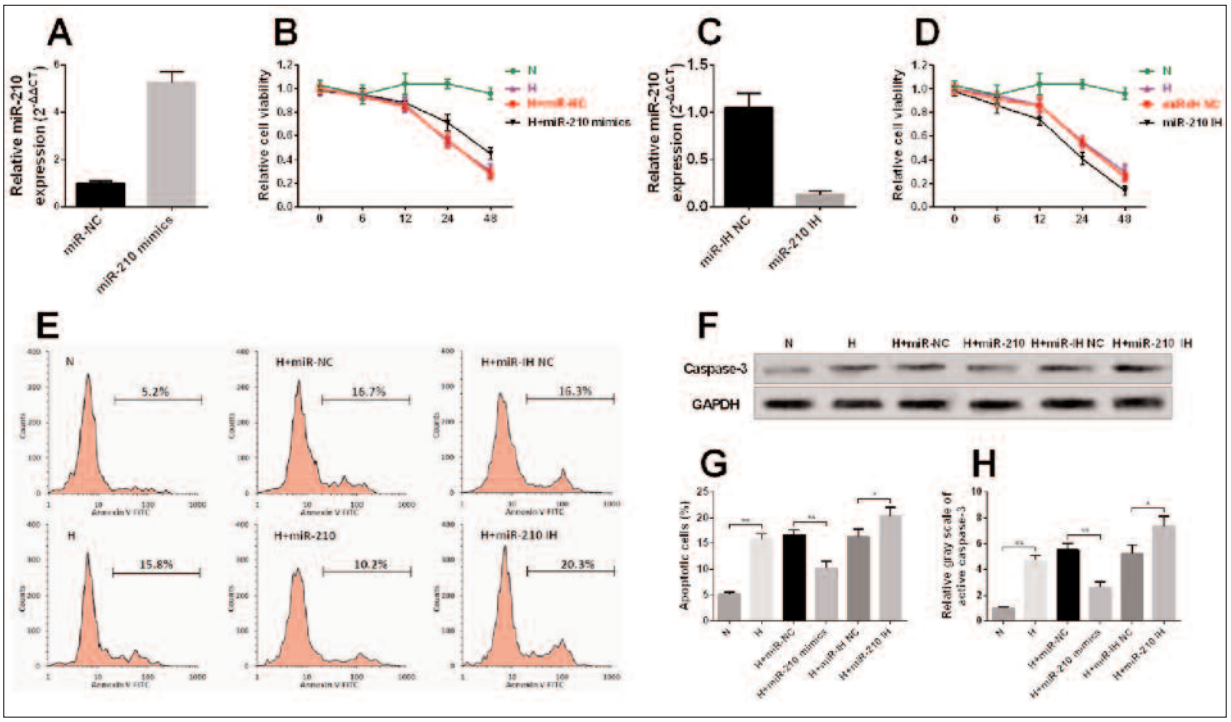

Figure 2. MiR-210 increases cell viability and reduces cell apoptosis of endometriotic cells in hypoxia. ***A*** and ***C,*** QRT-PCR analysis of miR-210 expression in CRL-7566 cells transfected with miR-210 mimics ***(A)*** or miR-210 inhibitor (IH) ***(C)***. ***B*** and ***D,*** CCK-8 assay of cell viability of CRL-7566 cells in normoxic and the cells with miR-210 overexpression ***(B)*** or knockdown ***(D)*** in hypoxic culture up to 48 hours. ***E,*** Flow cytometry analysis of apoptotic CRL-7566 cells with indicating treatments 24 hours after normoxic or hypoxic culture. ***F,*** Western blot analysis was further performed to detect active caspase-3 in the cells 24 hours after normoxic or hypoxic culture. ***G*** and ***H,*** Quantification of apoptotic cells ***(G)*** and relative expression of caspase-3 ***(H)*** showed in figure E and F. H: hypoxia; N: normoxia. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

miR-210 significantly attenuated hypoxia-induced apoptosis in CRL-7566 cells, while miR-210 inhibitor enhanced the apoptosis (Figure 2E and G). Following Western blot analysis also confirmed that miR-210 significantly attenuated hypoxia-induced activation of caspase-3, while miR-210 inhibitor enhanced the activation (Figure 2F and H). These results suggest that miR-210 increased cell viability and reduced cell apoptosis of endometriotic cells in hypoxia.

MiR-210 Enhances Autophagy of Endometriotic Cells in hypoxia at Least Partly Through Bcl-2

Autophagy usually acts as a natural pro-survival mechanism of cells in hypoxia[25]. A recent study[19] suggest that autophagy is upregulated in ovarian endometriosis and acts as an integral part of endometriosis pathogenesis. Therefore, we studied how miR-210 modulates autophagy of endometriotic cells in hypoxia. By measuring LC3I/II and p62 expression, we confirmed that hypoxia-induced significantly higher level of autophagy in CRL-7566 cells (Figure 3A). Enforced miR-210 overexpression significantly promoted autophagy in hypoxia, which was reflected by increased LC3II and reduced p62 (Figure 3A). In contrast, miR-210 inhibition resulted in reduced autophagy in hypoxia (Figure 3A).

One recent study[26] reported that miR-210 can directly bind to Bcl-2 and modulate hypoxia-induced apoptosis of neuroblastoma cells. In fact, besides the role in cell apoptosis, previous studies[20] also reported that Bcl-2 is involved in regulation of autophagy. Considering the verified role of miR-210 in autophagy of endometriotic cells in hypoxia, we further explored how miR-210/Bcl-2 axis involved in the regulation. CRL-7566 cells were firstly transfected with miR-210 mimics or Bcl-2 si-RNA (Figure 3B). Under hypoxic culture, knockdown of endogenous Bcl-2 significantly enhanced autophagy, the effect of

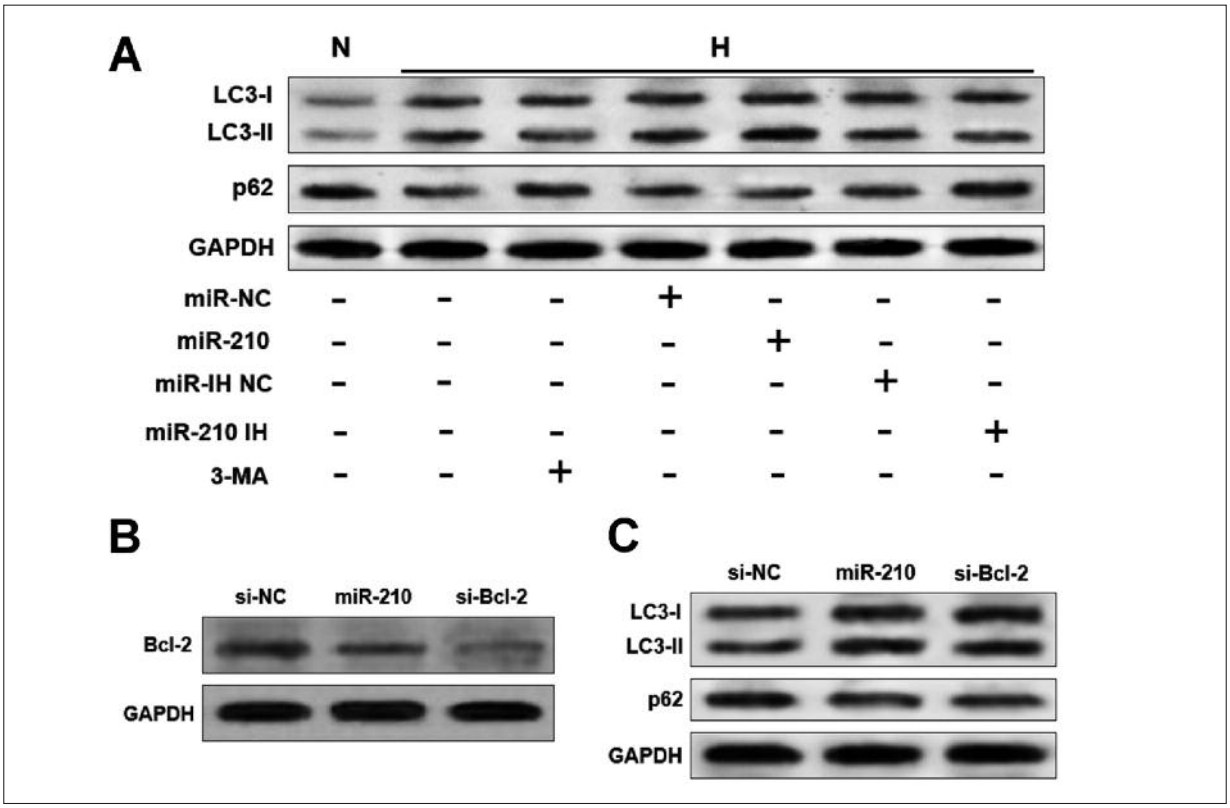

Figure 3. MiR-210 enhances autophagy of endometriotic cells in hypoxia at least partly through BCL-2. ***A,*** Western blot analysis of LC3-I/II and p62 expression in CRL-7566 cells in normoxia or in CRL-7566 cells with miR-210 overexpression or knockdown after 24 hours of hypoxic culture. ***B,*** Western blot analysis of Bcl-2 expression in CRL-7566 cells after 48 hours after transfection of miR-210 mimics or Bcl-2 siRNA. ***C,*** Western blot analysis of LC3-I/II and p62 expression in CRL-7566 cells with miR-210 overexpression or Bcl-2 knockdown 24 hours after hypoxic culture. H: hypoxia; N: normoxia. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

which was similar to that of miR-210 (Figure 3C). These results suggest that miR-210 enhanced autophagy of endometriotic cells in hypoxia at least partly through inhibiting Bcl-2.

Discussion

Although the etiology of endometriosis has not been fully revealed, there are emerging evidence showing that dysregulated of miRNA may play a role in the pathological processes[14,15]. A series of previous studies[16,24,27] reported that HIF-1±, the master transcription factor in response to hypoxia, is significantly increased in ectopic endometriotic tissue. HIF-1α can facilitate gene transcription through binding to the hypoxia response element (HRE) in the promoter regions of some genes. Through this mechanism, HIF-1α can modulate expression of multiple genes and therefore affect their downstream regulations. Previous studies reported that HIF-1α can bind to the promoter region of miRNAs, thereby enhancing their transcription. For example, the elevated HIF-1α expression in ectopic endometriotic tissue induces significantly higher miR-20a expression, which help maintenance of ectopic endometriotic lesions through enhancing dual-specificity phosphatase-2 controlled angiogenesis under the hypoxic environment[16,27].

MiR-210 has been reported as a hypoxia responsive miRNA in multiple types of cancer and can modulate their radiosensitivity[20,28,29]. In renal tumor, miR-210 is a target of HIF-1 and 2[22]. In this study, we confirmed that elevated HIF-1α expression in ectopic endometriotic tissue is associated with increased miR-210 expression. In *in-vitro* model based on ovarian endometriotic cell line, we also observed hypoxic culture increased HIF-1α and miR-210. In endometriotic cyst stromal cells, miR-210 transfection resulted in increased cell proliferation, higher expression of vascular endothelial cell growth factor (VEGF) and reduced cell apoptosis via STAT3 activation[23]. Therefore, miR-210 overexpression is involved in the pathogenesis of endometriosis-specific cellular dysfunctions through epigenetic mechanisms[23]. By performing CCK-8 assay and flow cytometry analysis of apoptosis, we observed that miR-210 enhanced cell viability and reduced cell apoptosis under hypoxia.

Autophagy acts as a spontaneous pro-survival mechanism of cells in hypoxia[25]. Through degrading cytoplasmic organelles and macromolecules, the basic components resulting from lysosomal digestion are then reutilized for anabolic metabolism[25]. This process plays a critical role in maintaining cellular homeostasis in both normal conditions or to overcome stress-induced conditions[25]. One recent study[19] reported that the autophagic response may also contribute to the cell survival and lesion maintenance of endometriosis. In addition, upregulated autophagy was also observed in ovarian endometriosis[19]. In this study, we observed that in endometriotic cells, miR-210 can promote autophagy in hypoxia. Therefore, miR-210 mediated autophagy may contribute to enhanced survival of endometriotic cells in hypoxia. Then, we performed preliminary studies to investigate the downstream regulator involved. Bcl-2, an anti-apoptotic protein, is a verified target of miR-210 in multiple cancers[20,26]. Previous study[30] found that Bcl-2 can suppress autophagy through binding to Beclin-1. Therefore, Bcl-2 may also act as an anti-autophagy protein via its inhibitory interaction with Beclin 1[30]. In this study, we found that miR-210 significantly inhibited Bcl-2 expression in endometriotic cells. In addition, Bcl-2 knockdown presents similar effect as miR-210 in promoting autophagy of endometriotic cells in hypoxia. This finding is consistent with one recent study[19], which reported that Bcl-2 was significantly downregulated in ectopic endometrial tissues compared with eutopic endometrial tissues. Therefore, it is conceivable that decreased expression of Bcl-2 due to elevated miR-210 in hypoxia disengage Beclin-1 to sustain the autophagic process of endometriotic cells.

Conclusions

The hypoxia-induced higher miR-210 expression may contribute to pathological development of endometriosis at least through enhancing cell survival and promoting autophagy via Bcl2/Beclin-1 axis.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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