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MicroRNA-135b suppresses extravillous trophoblast-derived HTR-8/SVneo cell invasion by directly down regulating *CXCL12* under low oxygen conditions

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ABSTRACT

The expression of numerous microRNAs (miRNAs) in the trophoblasts changes under low oxygen conditions. However, little is known regarding the regulation of the trophoblast invasion by miRNAs under low oxygen conditions. The aim of this study was to identify those miRNAs and their target genes associated with the trophoblast invasion under low oxygen conditions. Culturing the extravillous trophoblast (EVT) cell line, HTR-8/SVneo, at 2% oxygen as compared to 20% oxygen suppressed trophoblast invasion that correlated with increased expression of microRNA-135b (miR-135b) and decreased expression of the its predicted target gene *CXCL12*. Overexpression of miR-135b suppressed *CXCL12* mRNA expression and invasion of HTR-8/SVneo cells. Adding a neutralizing antibody against CXCL12 to the culture medium suppressed HTR-8/SVneo cell invasion. Reporter assays showed that the 3′UTR sequence of *CXCL12* was directly targeted by miR-135b. Our results suggest that miR-135b and *CXCL12* play important roles in modulating the EVT invasion under low oxygen conditions.

1. Introduction

Extravillous trophoblast (EVT) cells of the human placenta occlude the uterine spiral arterioles during the early first trimester [1]. The restriction of maternal blood flow provides a low oxygen environment in the intervillous space (IVS), which might be physiological and necessary for protecting an embryo against oxidative stress during early pregnancy [2]. Subsequent loss of these endovascular trophoblast plugs in the spiral arterioles after 10–12 weeks of gestation results in increasing maternal blood flow into the IVS and a dramatic increase in its oxygen tension [3]. This temporospatial modulation of the oxygen tension must be a very important alteration since the oxygen environment is one of the key regulators of trophoblast differentiation and determines optimal trophoblast proliferation and invasion.

Low oxygen tension appears to prevent the trophoblast differentiation towards an invasive phenotype [4,5]. Insufficient trophoblast invasion into the endometrium subsequently results in impaired vascular remodelling of the uterine spiral arterioles. Ultimately, the poor placentation after these events has been implicated in several complications of the pregnancy, including preeclampsia (PE) [6,7]. In spite of possible importance of local oxygen concentration for modulating the trophoblast invasion, the precise mechanisms that underlie the effects of low oxygen conditions on trophoblast invasion remain to be investigated.

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microRNAs (miRNAs) are endogenous small non coding RNAs that are important for regulating many cellular processes [8,9]. The expression of numerous miRNAs has been demonstrated in the human placenta [10], and abnormal miRNA expression levels have been reported in the placentas of the pregnancy disorders, including preeclampsia (PE) [11]. It was also reported that the expression of numerous miRNAs in the human placental trophoblasts changed after their exposure to low oxygen conditions [12]. These findings suggested that miRNAs might play key roles for phenotypical changes of the human trophoblast cells. However, little is known regarding the changes in the human trophoblast phenotypes due to miRNAs under the different oxygen concentration conditions.

Thus, in this study we sought to identify those miRNAs and their target genes in the trophoblasts that were associated with the cell invasion in response to changes in the oxygen concentration.

2. Materials and methods

2.1. EVT derived HTR-8/SVneo cell culture and oxygen concentration control

An immortalised first trimester EVT cell line (HTR-8/SVneo cells) was used for all the experiments. HTR-8/SVneo cell lines were kindly provided by Dr. Charles H. Graham (Queen's University, Ontario, Canada) and Dr. Eiko Yamamoto (Nagoya University Graduate School of Medicine, Nagoya, Japan) [13]. Cells were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 5% FBS (Sigma–Aldrich), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Nacalai Tesque) at 37 °C in a 5% CO_2 atmosphere. To treat the cells while under 2% O_2 conditions, we used a hermetically enclosed incubator with computerized monitoring sensor of atmospheric oxygen that was continually flushed with 5% CO_2 balanced nitrogen. We treated cells while under low oxygen conditions (2% O_2) or 20% O_2 conditions as a control for each experiment to determine the effects of a low oxygen concentration on EVT phenotypes.

2.2. Total RNA isolation

Total RNA from cultured HTR-8/SVneo cells was extracted using a miRNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The quality of the extracted RNA was confirmed using a NanoDrop spectrophotometer (NanoDropTechnology, San Diego, CA, USA) by measuring the absorbance at 230 nm, 260 nm and 280 nm.

2.3. miRNA expression profiles

miRNA microarray analysis was performed using a miRNA microarray (G4872A Human Rel. 16.0, Agilent Technologies, Santa Clara, CA, USA), which included 60 K probes for 1205 human and 144 viral RNAs based on Sanger miRBase Release 16.0. Total RNA samples extracted using the same experimental conditions for the three independent experiments were mixed equally before miRNA labelling for miRNA microarray experiments. miRNA labelling, hybridisation and washing steps were performed using a miRNA Complete Labelling and Hybridization Kit (Agilent Technologies). Each RNA sample (100 ng) was hybridised onto the miRNA microarray. Hybridisation, washing, staining and scanning were performed using Agilent Technologies system instruments and protocols. After hybridisation for 20 h, signals were detected with a microarray scanner (G2505B; Agilent Technologies) and quantified using Feature Extraction ver. 10.7.3.1 software (Agilent Technologies).

2.4. mRNA expression profiles

Total RNA samples used in miRNA microarray experiments were also used for mRNA microarrays. Total RNA samples were reverse transcribed, amplified and labelled using a GeneChip® 3′IVT Expression Kit (Affymetrix Inc., Santa Clara, CA, USA). Then, the resulting labelled complementary RNA (cRNA) was purified and fragmented, according to the manufacturer's instructions. cRNA samples together with the probe array controls were hybridised onto an Affymetrix GeneChip® Human Genome U133 Plus 2.0 array. Hybridisation, washing, staining and scanning were performed using Affymetrix GeneChip® system instruments and protocols. Scanned data were normalised using the Robust multi array average method.

2.5. Quantitative RT-PCR for miRNAs

To validate our microarray data, pooled total RNA samples extracted using the same experimental conditions for the three independent experiments were subjected to quantitative RT-PCR. Briefly, cDNA was synthesised from 5 ng of the total RNA isolated from HTR-8/SVneo cells with a TaqMan microRNA RT kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was done in duplicate with Mx3000P (Stratagene, La Jolla, CA, USA) using TaqMan Universal Master Mix II with UNG. TaqMan MicroRNA assays (Applied Biosystems) for miR-135b specific (Assay ID: 002261; Applied Biosystems) and endogenous control RNU48 specific (Assay ID: 001094; Applied Biosystems) primers were used for reverse transcription and RT-PCR analysis. RNU48 was selected as an endogenous control based on the background experiments. The mature sequences of hsa-miR-135b and RNU48 were 5′-UAUGGCUUUUCAUUCCUAUGUGA-3′ (miRBase Accession number: MIMAT0000758) and 5′ GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTGACC-3′ (NCBI Reference Sequence: NR_002745), respectively. miR-135b expression levels, relative to RNU48 were determined using the $2^{-\Delta\Delta ct}$ method.

2.6. Quantitative RT-PCR for mRNAs

Pooled total RNA samples extracted using the same experimental conditions for the three independent experiments were used when validating our microarray data. Briefly, the total RNA was reverse transcribed using BioScript Reverse Transcriptase (Bioline, London, UK) with oligo dT primers, according to the manufacturer's instructions. Using the resulting cDNAs as templates, the gene expression levels were determined with an Mx3000P system (Stratagene, La Jolla, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. The primer sequences used for mRNA expression were: 5′-AGAGCTACGAGCTGCCTGAC 3′ (actin, beta (ACTB) forward); 5′-AGCACTGTGTTGGCGTACAG-3′ (ACTB reverse); 5′-AGCCAACGTCAAGCATCTCA-3′ (chemokine (C-X-C motif) ligand12 (*CXCL12*) forward) and 5′-TAGCTTCGGGTCAATGCACA-3′ (*CXCL12* reverse). *CXCL12* expression levels, relative to *ACTB* were determined using the $2^{-\Delta\Delta ct}$ method.

2.7. Search for candidate miRNAs and predicted target genes by ingenuity pathways analysis (IPA)

miRNA and mRNA microarray data were analysed using the microRNA target filter in IPA software (Ingenuity® Systems; www.ingenuity.com) to identify the potential mRNA targets of miRNAs. The data for the differentially expressed mRNAs were integrated with miRNA expression data, using the IPA expression pairing

function to obtain miRNA-mRNA relationships that showed expression changes in the opposite directions.

2.8. Transwell cell invasion assay

A transwell cell invasion assay was done using the growth factor reduced (GFR) Matrigel coated invasion chambers in 24-well plates (Becton Dickinson, East Rutheford, NJ). Briefly, HTR-8/SVneo cells (5×10^4 cells/500 μl/well) in the serum free culture medium were plated on top of the Matrigel coated transwell inserts with 8-μm pores in the filter membrane. At the same time, 0.75 ml of the culture medium with 5% FBS was added to the lower compartment, after which the transwell containing plates were incubated for 24 or 48 h in a humidified 5% CO_2 atmosphere. After the incubation, noninvading cells were removed with a cotton swab. Invading cells that had entered the lower surface of the filter membrane were stained with Diff-Quick (Sysmex, Kobe, Japan). Cell invasion was quantified in a blinded manner by counting the numbers of the invading cells on the lower surface of the membrane in 5 fields (100 × magnification) per chamber. Each experiment was done more than 3 times using triplicate samples for each experiment.

2.9. Transfection experiments with miR-135b mimics

To promote miRNA activities, we used mirVana™ miRNA mimics (Applied Biosystems). The catalogue number for RNA reagent was MC13044 (hsa-miR-135b-5p mimic). We also used mirVana™ miRNA Mimic Negative Control #1 as a negative control for these reactions. For transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was mixed with 20 nM of each of the RNAs according to the manufacturer's protocols. Then, these solutions were placed into each well prior to the transfection and HTR-8/SVneo cells were added directly to each well at a density of 60% confluence well in 12-well plates. After incubation for 24 or 48 h, we performed functional analyses of miRNAs.

2.10. CXCL12 neutralizing antibody treatment

We used a mouse anti human/mouse CXCL12/SDF-1 monoclonal antibody (MAB310; R&D Systems, Abingdon, UK) to neutralise CXCL12. We also used mouse immunoglobulin (Ig) G1 Isotype Control (MAB002; R&D Systems) as a negative control. HTR-8/SVneo cells were incubated with each antibody (40 μg/ml) for each experiment. The working concentration of CXCL12 neutralizing antibody was determined according to the manufacturer's instructions and the previous study reported by Zhou et al. [14].

2.11. Luciferase reporter assay

To found the putative miR-135b seed sequence of the *CXCL12* 3′UTR, we performed bioinformatics analysis using Tagetscan, miRanda and PicTar. The miR-135b putative target region of the *CXCL12* 3′UTR sequence we found by aforementioned bioinformatics analysis (5′-ATATATTTGAAGTGGAGCCATA-3′, part of the NCBI Ref Seq ID of NM_199168) or its mutated sequence (5′-ATATATTTGAAGTGGCCGGCCC-3′) was cloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) using *Sac*I and *Xba*I sites. The resulting constructs were designated pmirGLO-CXCL12 and pmirGLO-CXCL12-mut, respectively. Insertions were confirmed by sequencing both DNA strands. Co-transfection of 200 ng of pmirGLO-CXCL12 or pmirGLO-CXCL12-mut with 1.0 nM hsa-miR-135b-5p mimic or negative control (mirVana™ miRNA Mimic Negative Control #1) was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions in a 24-well plate. At 48 h post transfection, transfected cells were washed with cold PBS, lysed using 1 × passive lysis buffer (Promega) and assayed for firefly and *Renilla* luciferase activities with a dual luciferase reporter assay system (Promega), according to the manufacturer's instructions using an ARVO MX plate reader (Perkin–Elmer). Relative firefly luciferase activity was determined by normalisation to *Renilla* luciferase activity. Each experiment was done four times using triplicate samples for each experiment.

2.12. Statistical analyses

Results are given as means ± SD's. Statistical comparisons were made using Student's t-tests. A p value of <0.05 was considered significant. Statistical analyses were performed using Statcel 2 for Windows (OMS, Tokyo, Japan).

3. Results

3.1. HTR-8/SVneo cell invasion under low oxygen conditions

We investigated the effect of the oxygen concentration on EVT cell invasion. HTR-8/SVneo cells showed significantly reduced numbers of the invading cells after 48 h under 2% O_2 as compared with 20% O_2 (Fig. 1A). This result suggested that a low oxygen concentration (2% O_2) suppressed the invasion of HTR-8/SVneo cells.

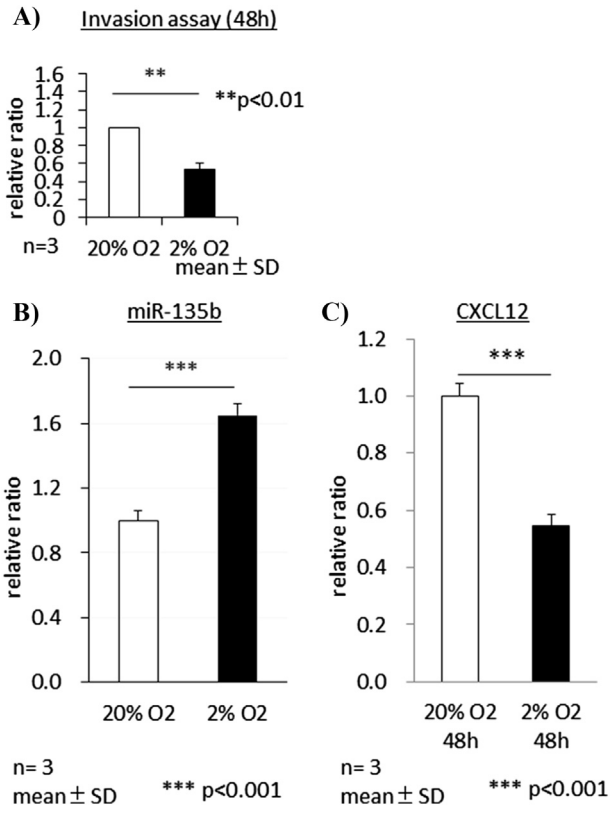

Fig. 1. HTR-8/SVneo cell invasion and real time RT-qPCR analysis for miR-135b and *CXCL12* while under 2% O_2 low oxygen conditions A) Cell invasion was significantly inhibited after culture for 48 h under the 2% O_2 low oxygen conditions as compared with 20% O_2 conditions. B) miR-135b expression was up-regulated 1.65 fold under 2% O_2 as compared with 20% O_2. C) *CXCL12* expression was decreased to 0.54 fold under 2% O_2 as compared with 20% O_2. Error bars are standard deviations. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. miRNA expression microarray results for HTR-8/SVneo cells under low oxygen conditions

To identify miRNAs that might be related to the phenotypic changes of EVT cell invasion under low oxygen conditions, HTR-8/SVneo cells were incubated under low oxygen conditions (2% O_2) or 20% O_2 conditions as a control for 48 h. Subsequently, we extracted the total RNAs from these cells and used these for miRNA microarray experiments. We excluded as candidates those miRNAs for which their log2-transformed signals on the miRNA microarray were <3.5 under the higher expression in 2% or 20% O_2 conditions. Table 1 showed that 10 miRNAs were found to have the expression changes of at least 1.8 fold in either direction under 2% O_2 conditions compared with those under 20% O_2 conditions. There were five up-regulated and five down-regulated miRNAs under 2% O_2 conditions.

3.3. Candidate miRNAs and predicted target genes related to EVT cell invasion under low oxygen conditions

To determine the target genes of the miRNAs associated with EVT cell invasion under low oxygen conditions, we also conducted mRNA expression microarray analysis with the same total RNA samples used for miRNA microarray analysis. The 10 differentially expressed miRNAs shown in Table 1 were analysed using the microRNA target filter in IPA to identify their potential mRNA targets. The mRNA expression microarray data were integrated with those of the 10 indicated miRNAs using the expression pairing functions of IPA to obtain miRNA-mRNA relationships of which the expression changes in the opposite directions.

Among the 10 miRNAs shown in Table 1 and their predicted target genes in the IPA analysis, we were interested in miR-135b and its predicted target gene *CXCL12* for the further analysis. *CXCL12* expression was decreased to 0.41 fold under 2% O_2 as compared with 20% O_2. *CXCL12* is one of the known genes that promotes EVT cell invasion [14,15]. The function of miR-135b in EVT cells, however, is poorly understood. Thus, we focused on evaluating a possible regulatory role of miR-135b against *CXCL12*.

3.4. Real-time RT-qPCR for miR-135b and CXCL12

To validate our miRNA and mRNA microarray data, miR-135b and *CXCL12* expressions were further confirmed by real time RT-qPCR. As shown in Fig. 1, the miR-135b/RNU48 ratios in HTR-8/SVneo cells under 2% O_2 as compared with 20% O_2 were significantly up-regulated. In contrast, the *CXCL12*/*ACTB* ratios under 2% O_2 as compared with 20% O_2 were significantly down-regulated. These real time RT-qPCR results for miR-135b and *CXCL12* expression were consistent with those obtained from our microarray analysis.

Table 1
Differentially expressed miRNAs in HTR-8/SVneo cells with fold changes of >1.8 in either direction while under 2% O_2 compared to 20% O_2 for 48 h.

Up-regulated in 2% O_2				Down-regulated in 2% O_2			
miRNA	[a]Signal in 2% O_2	[a]Signal in 20% O_2	[b]Fold change	miRNA	[a]Signal in 2% O_2	[a]Signal in 20% O_2	[b]Fold change
miR-210	9.40	6.53	7.30	miR-4417	3.87	4.90	0.49
miR-203	3.83	2.95	1.85	miR-3656	3.52	4.54	0.49
miR-30b-5p	8.82	7.95	1.83	miR-7-5p	3.54	4.52	0.51
miR-30d-5p	7.42	6.57	1.81	miR-663a	3.85	4.71	0.55
miR-135b	3.84	2.98	1.81	miR-33b-3p	2.75	3.60	0.56

[a] Signal values are log2-transformed signals from the microarray experiments.
[b] Fold changes are fold changes for 2% O_2/20% O_2.

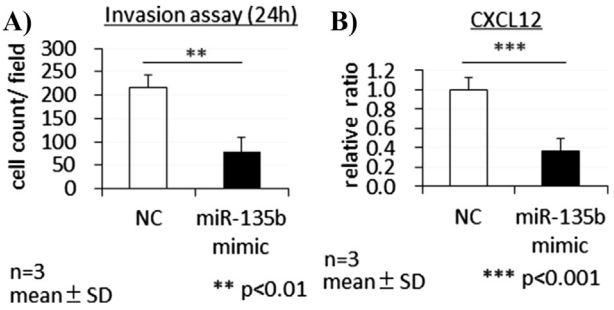

Fig. 2. Effects of miR-135b overexpression in HTR-8/SVneo cells A) Cell invasion was significantly suppressed after the transfection with a miR-135b mimic. B) *CXCL12* expression was significantly suppressed after the transfection with a miR-135b mimic. Error bars are standard deviations. NC; negative control, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5. miR-135b overexpression results

To explore the functional role of the miR-135b on the expression of predicted target gene *CXCL12* and on EVT cell invasion, HTR-8/SVneo cells were transfected with a miR-135b mimic or its control, followed by the cell invasion assays and real time RT-qPCR assay for *CXCL12* expression. Cell invasion assays were started at 24 h after miRNA mimic transfection and the amount of cell invasion was quantified after incubating the transfected cells for further 24 h. Cell invasion was significantly reduced when miR-135b mimic was overexpressed and *CXCL12* mRNA expression was also significantly down-regulated (Fig. 2A,B).

3.6. HTR-8/SVneo cell invasion after the treatment with an anti-CXCL12 neutralizing antibody

Two reports showed that *CXCL12* in the first trimester human trophoblasts promoted their own invasiveness [14,15]. Thus, to explore the functional role of *CXCL12* in this cell line, we conducted the cell invasion assays with HTR-8/SVneo cells using an anti-CXCL12 neutralizing antibody. In each cell invasion assay, cells that were placed on the upper transwell inserts were treated with an anti-CXCL12 neutralizing antibody (40 μg/ml) or a mouse IgG1 isotype control (40 μg/ml), followed by the quantification of the cell invasion after incubating for 24 h. Invasion by HTR-8/SVneo cells was significantly reduced after the treatment with the anti-CXCL12 neutralizing antibody (Fig. 3).

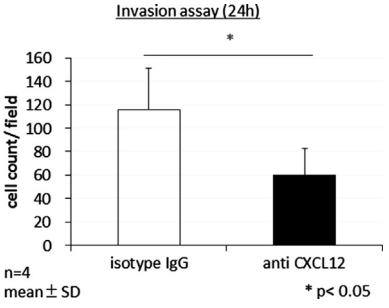

Fig. 3. HTR-8/SVneo cell invasion after the treatment with an anti-CXCL12 neutralizing antibody HTR-8/SVneo cell invasion was significantly reduced after treatment with an anti-CXCL12 neutralizing antibody (40 μg/ml). Error bars are standard deviations. Isotype IgG; Mouse immunoglobulin (Ig) G1 Isotype Control antiCXCL12; mouse Anti-Human/Mouse CXCL12/SDF-1 monoclonal antibody $^{*}P < 0.05$.

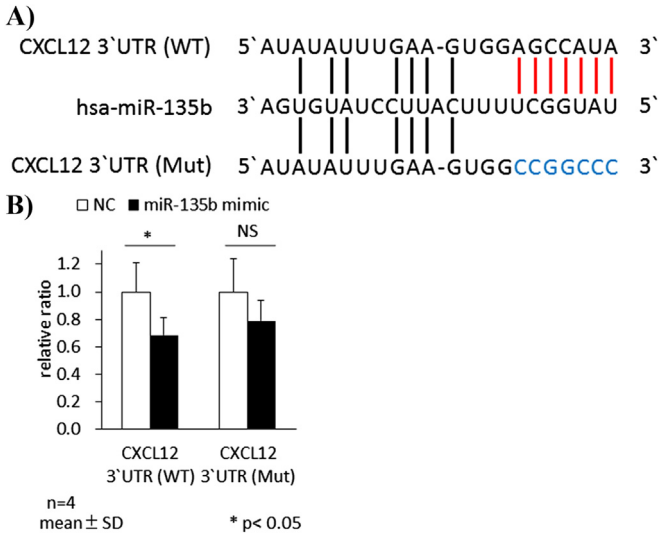

Fig. 4. Luciferase reporter assay to test whether *CXCL12* was a direct target gene of miR-135b A) Sequence alignment found in bioinformatics analysis (Tagetscan, miRanda, PicTar) between miR-135b and its putative binding site in the *CXCL12* 3′UTR. B) The relative luciferase activity was reduced by 32.5% when the *CXCL12* 3′UTR reporter construct with a wild type target site was co-transfected with a miR-135b mimic. When the target sequence of *CXCL12* was mutated, luciferase activity did not significantly change. Error bars are standard deviations. WT; wild-type, Mut; mutated type, NC; negative control, $^{*}P < 0.05$, *NS*; not significantly different.

3.7. miR-135b directly regulates the predicted target site in the 3′UTR of the CXCL12 gene

As a predicted direct target of miR-135b, we found the specific site in the 3′UTR of the *CXCL12* by bioinformatics analysis using Tagetscan, miRanda and PicTar (Fig. 4A). We used luciferase reporter assays to determine whether *CXCL12* was a direct target of miR-135b. To test whether the abovementioned predicted target sequence in the 3′UTR of the *CXCL12* gene was directly regulated by miR-135b, we cloned the predicted target sequence downstream of the luciferase reporter gene and co-transfected it with a miR-135b mimic. The relative luciferase activity was reduced by 32.5% when the *CXCL12* 3′UTR reporter construct with wild type sequence was co-transfected with a miR-135b mimic (Fig. 4B). When the targeted sequence of *CXCL12* was mutated (Fig. 4A), the luciferase activity did not change significantly (Fig. 4B). These results suggested that miR-135b directly regulated the predicted target site in the 3′UTR of the *CXCL12* gene.

4. Discussion

It is known that miR-135b is abundantly expressed in human term placentas [16] and is also detectable in an isolated trophoblast cells from the first trimester villous [17]. In cancer research, it is also known that miR-135b is associated with colorectal, lung and thyroid cancer cell invasion and promotes the cancer progression by directly modulating the actions of several genes [18–20]. Together with our findings, a role for miR-135b to modulate the trophoblast invasion during the placenta formation is compelling. To the best of our knowledge, this is the first report to demonstrate that miR-135b is involved in the phenotypical changes of the trophoblasts in a low oxygen condition. Because the modulation of oxygen concentration during the trophoblast differentiation into an invasive or proliferative phenotype is an important factor for the placenta formation [4,5], miR-135b may be crucial for regulating the trophoblast phenotypes during the first trimester.

Because the target genes of miR-135b in the trophoblast biology had not been identified, we used the microRNA target filter in IPA to search for their potential mRNA targets. We confirmed that miR-135b directly regulated *CXCL12* gene expression using a luciferase reporter assay. *CXCL12* gene expression is induced by HIF-1α in fibroblasts and endothelial cells under low oxygen conditions [21,22], whereas the low oxygen stress inhibits *CXCL12* expression in the human uterine endometrial stromal cells in a HIF-1α-independent manner [23]. Our current study suggested that the inhibition of *CXCL12* gene expression by miR-135b is a novel regulatory system that could influence the cell invasion capability under low oxygen conditions as with uterine endometrial stromal cells. This regulatory system may be important for EVT invasion into maternal decidua, myometrium, or spiral arteries and impaired EVT invasion causes inadequate vascular remodelling and subsequent poor placentation. However, the precise mechanism underlying *CXCL12* inhibition under low oxygen conditions in this EVT cell line remains to be determined.

In primary cultures of villous explants from the first trimester, these cells' invasiveness was inhibited under low oxygen conditions [24]. In contrast, a consensus has not been reached on the behaviours of HTR-8/SVneo cells under low oxygen conditions. Several reports showed opposing results regarding the invasiveness of this cell line. Graham et al. reported that the cell culture under the 1% O_2 low oxygen conditions promoted the cellular invasion [25]. In contrast, Kilburn et al. reported that low oxygen conditions reduced the invasiveness of HTR-8/SVneo cells [26]. Additionally, Lash et al. reported that HTR-8/SVneo cells' invasiveness increased after the culture for 24 h but decreased after 72 h under the 3% O_2 low oxygen conditions [27]. In this study, HTR-8/SVneo cells' invasiveness was suppressed under 2% O_2 conditions for 48 h. We assume that these different results were due to the difference in oxygen concentrations used, culture periods, the presence of serum, cell seeding densities, and the methods used for the invasion assays. Because all of the experiments in this study were done using only one cell line, additional studies will be needed to determine the physiological and the pathological roles of miR-135b and *CXCL12* during the placentation *in vivo*. We also need to validate the potential use of miR-135b as a therapeutic target or a diagnostic marker for the pregnancy complications related to poor placentation.

In conclusion, our findings suggest that miR-135b suppresses the invasiveness of HTR-8/SVneo cells by directly down regulating *CXCL12* expression under low oxygen conditions. We propose that miR-135b and *CXCL12* play an important role in modulating the invasion of EVT cells during the first trimester.

Conflict of interest

None.

Acknowledgments

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