Promotion of trophoblast invasion by IncRNA MVIH through inducing Jun-B

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Abstract

Preeclampsia (PE), a pregnancy-specific disorder, is associated with impaired uterine spiral artery remodelling, which is related to the dysfunction of trophoblast cells. Lately, mounting evidence has indicated that aberrant expression of long non-coding RNAs (lncRNAs) is associated with various human diseases. The lncRNA MVIH transcript has been shown to decrease the severity of several diseases. However, the biological function of MVIH, which is down-regulated in placental tissues in PE, has not yet been clarified. Here, we report that MVIH may act as a vital factor in the pathogenesis of PE. In this study, functional analysis revealed that the silencing of MVIH expression via transfection with small interfering RNA (siRNAs) inhibited cell growth, migration, invasion, and angiogenesis in various trophoblast cell lines, and stimulation with MVIH could promote these functions. Mass spectrometry analysis revealed that MVIH could modulate Jun-B protein expression, which has been reported to potentially regulate cell growth and angiogenesis. Further cotransfection assays were performed, revealing that MVIH and Jun-B have a synergistic effect on the regulation of angiogenesis and cell proliferation. Taking these findings together, MVIH could be associated with PE and may be a candidate biomarker for its diagnosis and treatment.

Keywords: long non-coding RNAs • MVIH • Preeclampsia • Jun-B

Introduction

Preeclampsia (PE) is a pregnancy-specific disease characterized by hypertension and proteinuria [1]. It remains a leading cause of maternal mortality and morbidity worldwide [2]. Shallow extravillous trophoblast (EVT) invasion and impaired spiral artery remodelling are thought to be involved in the pathogenesis of PE [3]. Trophoblast dysfunctions, such as inhibited proliferation [4], anti-angiogenesis [5] and decreased migration and invasion [6], make major contributions to the failure of spiral artery remodelling. Hence, to deepen our understanding of this disease, it is important to evaluate the molecules underlying the biological functions of trophoblasts.

With rapid innovations in whole-genome sequencing techniques, the significance of non-coding RNAs (ncRNAs) for a range of biological processes has been determined, particularly that of long non-coding RNAs (lncRNAs) [7–9]. lncRNAs are a newly identified class of non-coding RNAs that are more than 200 nt in length with limited or no protein-coding capacity. The human genome contains approximately 7000–23,000 lncRNAs [10]. Multiple lines of evidence have revealed the contribution of lncRNAs to a wide spectrum of biological processes, including the induction of disease, X-chromosome inactivation in embryonic development, chromatin modification in substance metabolism and the dysfunction of cells in tumorigenesis [11–14]. Moreover, accumulating evidence indicates that deregulated IncRNAs are closely related to the pathogenesis of PE [15, 16].

Our previous studies also revealed a panel of IncRNAs that might serve as potential biomarkers for predicting PE [17, 18]. Among these,
abnormal expression of SPRY4-IT1, a highly conserved lncRNA that is localized in the nucleus, might be predictive of trophoblast metastasis and apoptosis in PE [17]. Findings also suggested that down-regulated lncRNA MEG3 in PE might account for the promotion of trophoblast apoptosis and suppression of trophoblast invasion [18]. Moreover, after screening for IncRNAs that exhibited differential expression between PE placentas and normal cases, another IncRNA MVIH (microvascular invasion in hepatocellular carcinoma), was examined.

MVIH is an IncRNA that was only recently identified; it was shown to have the potential to modulate angiogenesis and cell invasion [19]. The significantly lower level of MVIH in PE placentas and normal cases, another lncRNA MEG3, may be a latent biomarker for PE diagnosis [20].

Materials and methods

Tissue samples and clinical data collection

Thirty paired placental tissues from women with PE and controls were obtained, after which all obtained tissue samples were instantly snap-frozen in liquid nitrogen and stored at −84°C before RNA extraction. The detailed clinic characteristics of the patients are recorded in Table 1. Our study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, China. Written informed consent was obtained from all of the subjects in this study.

Cell culture

HTR-8/SVneo, an immortalized first-trimester EVT cell line [20] that originated from a short-lived primary EVT cell line, was generously provided by Dr. Charles Graham, Queen’s University, Canada. With the behaviour of trophoblast cells, many studies [21, 22] have used this cell line to simulate trophoblast cells in pregnancy.

Human choriocarcinoma cells (JEG-3) and human umbilical vein endothelial cells (HUVEC) were obtained from the Chinese Academy of Sciences Committee (Shanghai, China). HTR-8/SVneo, JEG-3 and HUVEC-C were incubated in RPMI 1640 (Gibco, Nanjing, China), DMEM (Gibco, Nanjing, China), and ECM (Gibco, Invitrogen, Carlsbad, CA, USA), respectively, which had been supplemented with 5% FBS (Gibco, Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin in humidified air at 37°C with 5% CO₂.

RNA preparation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA), and qRT-PCR analyses were performed in accordance with the manufacturer’s manual (Takara, Dalian, China). A reverse transcription kit (Takara) was used for the synthesis of cDNA. The amplification of cDNA was performed using Power SYBR Green (Takara) in a reaction mix for qRT-PCR with a total volume of 20 μl. In accordance with the manual, the reverse transcription was implemented at 37°C for 15 min, and 85°C for 5 sec. The qRT-PCR results were analysed and expressed relative to threshold cycle (CT) values, and then converted to fold changes. The related primer sequences and siRNAs are presented in Table S1.

Transfection of cell lines

HTR-8/SVneo, JEG3 and HUVEC-C cell lines were plated in six-well plates and then transfected with corresponding siRNAs (10 μM) or plasmid vectors (4 μg) using Lipofectamine 2000 (Invitrogen) and plasmid vectors, respectively. pRES2-EGFP and pRES2-MVIH were extracted using DNA Midiprep kit (Qiagen, Hilden, Germany). At 48 hrs after treatment, trophoblast cells were harvested for further experiments, as exemplified by qRT-PCR and Western blotting.

Cell proliferation assays

Cell viability was determined using the MTT assay (Cell Proliferation Reagent Kit I; Roche Applied Science, Penzberg, Germany). The cell lines were transfected with si-MVIH or pRES2-MVIH (3000 cells/well) and were plated in 96-well plates with five duplicates. Cell viability was tested every 24 hrs, in accordance with the instructions. The absorbance was detected at 490 nm with an ELx-800 University Microplate Reader (BioTek, Winooski, VT, USA).
Flow cytometry

Flow cytometry was performed to determine and quantify the phases of cells within the cell cycle. Cells for cell cycle analysis were stained with propidium oxide using the Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA), in accordance with the manufacturer’s manual, and analysed by FACScan. The proportions of cells in the G0–G1, S and G2–M phases were determined and compared.

Transwell assays

Cell migration and invasion abilities were analysed by Transwell assays. A total of $3 \times 10^4$ to $5 \times 10^4$ cells were plated on the top of a membrane precoated with Matrigel (BD Biosciences; without Matrigel for cell migration assays). Upon incubation for 24–48 hrs, cells inside the upper chamber were removed with cotton swabs, while cells on the lower membrane surface were fixed with methanol and then stained with 0.5% Crystal violet solution. Five randomly selected fields were counted in each well.

Network formation assay

Previous studies revealed that HTR-8/SVneo and HUVEC-C cells showed endothelial cell-like behaviour regarding their ability to form tube-like networks when grown on a Matrigel. The network formation assay was thus performed here to determine the ability of cells to undergo tube formation, as previously reported by Zou et al. [17]. A total of $2.5 \times 10^4$ cells treated with si-MVIH or pIRE2-MVIH were cultured in the upper layer of the chamber previously coated with Matrigel. After 6 hrs, images from five areas of each 96-well plate were captured using a fluorescent microscope.

Mass spectrometry analysis

To identify the relevant proteins targeted by MVIH, the bands that were particularly intense in the MVIH sample were selected for further analysis. These bands were excised for mass spectrometry analysis, which was performed by Shenzhen Genomics Institute (Shenzhen, China). Those proteins sequenced with at least two peptides were considered as a reliable identification. The detailed mass spectrometry data and information on protein identification are provided in Table S2.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 software (Armonk, NY, USA). The data are presented as the mean ± S.D. Statistical significance was assigned at $P < 0.05$ (*) or $P < 0.01$ (**) .

Results

MVIH expression is down-regulated in placental tissues of PE

To explore the biological function of MVIH in placental tissue samples in cases of PE, we first determined the expression levels of MVIH in 30 paired placental tissues from PE and normal pregnancies by performing quantitative PCR (qPCR). The levels of MVIH transcript were markedly down-regulated in PE placental tissues compared with their levels in placental tissue samples from normal pregnancies ($P < 0.01$; Fig. 1). The detailed clinical characteristics, including systolic blood pressure, diastolic blood pressure, proteinuria and foetal birth weight, are presented in Table 1. The results indicate that MVIH might be a vital predictive biomarker for PE patients.

Up-regulation and down-regulation of MVIH in trophoblast cell lines

To explore the biological function of MVIH in placental tissues of PE, we first determined the expression levels of MVIH in 30 paired placental tissues from PE and normal pregnancies by performing quantitative PCR (qPCR). The levels of MVIH transcript were markedly down-regulated in PE placental tissues compared with their levels in placental tissue samples from normal pregnancies ($P < 0.01$; Fig. 1). The detailed clinical characteristics, including systolic blood pressure, diastolic blood pressure, proteinuria and foetal birth weight, are presented in Table 1. The results indicate that MVIH might be a vital predictive biomarker for PE patients.

Down-regulation of MVIH inhibits trophoblast cell proliferation in vitro

At 48 hrs after transfection, we performed MTT assays and the resulting data revealed that the knockdown of MVIH expression considerably inhibited cell growth in HTR-8/SVneo and JEG-3 cells compared with that in the controls (Fig. 3A and C). Consistent with this, the stimulation of MVIH expression facilitated cell proliferation in HTR-8/SVneo and JEG-3 cells (Fig. 3B and D).
Effects of MVIH on the migration and invasion of trophoblast cell lines

The migration and invasion of trophoblast cells are crucial for the progression of diverse diseases, particularly PE [17]. Therefore, we investigated the effects of MVIH on the invasion and migration of trophoblast cells by performing Transwell assays. As shown in Figure 4A and B, the overexpression of MVIH stimulated cell migration. Conversely, the down-regulation of MVIH inhibited the migration ability of HTR-8/SVneo, with a considerable decline in the number of migratory cells (Fig. 4C and D). As shown in Figure 4E–H, silencing of MVIH impeded the invasion of JEG-3, while MVIH overexpression had the opposite effect. These results indicate that MVIH can promote the migration and invasion of trophoblast cells.

Effects of MVIH on network formation ability in vitro

Previous studies indicated that impaired spiral artery remodelling is involved in the pathogenesis of PE and resulted in placental ischaemia, hypoxia, and the release of a variety of placental factors into the maternal blood circulation [3, 23], which promoted activation of the systemic inflammatory response and vascular endothelial injury, in turn potentially leading to a systemic reaction. In this study, we investigated the influence of MVIH on the network formation ability of HUVEC and HTR-8/SVneo cells, and shed light on the involvement of MVIH in the pathogenesis of PE. As shown in Figure 5A, B, E and F, the overexpression of MVIH boosted the network formation ability of HTR-8/SVneo and HUVEC-C cells. Conversely, the knockdown of MVIH inhibited the network formation ability and considerably decreased the number of capillary-like networks, as revealed by the network formation assay in vitro (Fig. 5C, D, G, and H).

In addition, we determined the expression of vascular endothelial growth factor (VEGF) and its soluble vascular endothelial growth factor receptor-1 (sFlt-1), angiopoietin I and II (AngI and AngII) by qPCR after transfection with effective siRNAs versus si-NC and pIRES2-EGFP versus pIRES2-MVIH, respectively. The qPCR analysis revealed that the expression of VEGF, AngI and AngII was down-regulated to different degrees after MVIH knockdown while sFlt-1 was increased; conversely, the overexpression of MVIH promoted the expression of these genes in HTR-8/SVneo and HUVEC cells (Fig. 6I and J). These results indicate that MVIH might modulate the pathogenesis of PE by affecting the formation of vascular endothelial cells and the expression of angiogenesis-related factors.

Proteins regulated by MVIH as revealed by mass spectrometry

To assess further which proteins were associated with MVIH upon transfection with si-MVIH or pIRES2-MVIH, we then performed mass spectrometry analysis to identify proteins involved in the effects of MVIH in the trophoblast cell lines. As shown in Figure 6A, a total of 243 proteins were differentially expressed between the group with MVIH overexpression (pIRES2-MVIH) and the control group (pIRES2-EGFP), among which 114 were up-regulated and 129 down-regulated. There were 289 proteins that were differentially expressed after transfection with siRNAs compared with si-NC in HTR-8/SVneo cells, of which 172 showed increased and 117 decreased expression.

Data were obtained from clustering analysis performed separately on cells subjected to either down-regulation or overexpression of MVIH. Six proteins showed a significant difference with contrary tendency between the down-regulation and overexpression groups, respectively.
namely, BTAF1 (TATA-binding protein associated factor 1, Gene ID: 9044), H2A1 (Human Histone H2A type 1, Gene ID: 373337), PYC (Human pyruvate carboxylase in mitochondria, Gene ID: 5091), TK1 (Human Thymidine Kinase, Gene ID: 7083), RCL1 (RNA terminal phosphate cyclase-like 1, Gene ID: 10171) and JunB (Q6IBG3, Gene ID: 3726) (Fig. 6B). Screening revealed that JunB had particular potential to modulate angiogenesis and cell proliferation. Therefore, we focused on JunB for further investigation.

To confirm the involvement of Jun-B in the biological functions of MVIH, we performed cotransfection assays with pEGFP-JunB and si-MVIH in HTR/SVneo cells. MTT assays suggested that the
Fig. 4 Effects of MVIH on the migration and invasion of two trophoblasts cell lines. The migration and invasion capacity of the cells transfected with si-MVIH were significantly lower than that of the negative control and higher in the cells overexpressing pIRES2-MVIH, as determined by Transwell assays (Values are mean±S.D.; **P < 0.01) (A–H).

Fig. 5 Effects of MVIH on network formation ability in vitro. Cells transfected with siRNAs targeting MVIH showed an increase in node numbers as compared to the negative control while cells treated with plasmid overexpressing pIRES2-MVIH presented a decrease in node numbers as compared to that of the pIRES2-EGFP (A–H). The expression of relevant vascular endothelial growth factor (VEGF) by qPCR after transfecting with effective siRNAs and pIRES2-MVIH, respectively. (I and J) (Values are mean±S.E.M.; *P < 0.05; **P < 0.01).
overexpression of Jun-B facilitated the proliferation of HTR-8/SVneo cells, while the proliferative ability was considerably inferior in the group cotransfected with pEGFP-JunB and si-MVIH (Fig. 7A). The same trend was also found in the migration assays (Fig. 7B–E). The proliferation and migration of Jun-B could be counteracted by silencing MVIH, demonstrating that MVIH might play a direct regulatory role on Jun-B.

Next, we performed network formation assays to investigate the angiogenesis ability after cotransfection with pEGFP-JunB and si-MVIH in HUVEC cells. The results revealed that stimulation with Jun-B promoted angiogenesis ability; increased the expression of VEGF, AngI and AngII; and decreased the expression of sFlt-1 compared with the levels in the controls; the opposite trends were revealed after cotransfection with si-MVIH and pEGFP-Jun-B (Fig. 7F–K). These findings suggest that MVIH and JunB have a synergistic effect on the regulation of angiogenesis.

Discussion

Besides miRNAs, tens of thousands of lncRNAs derived from the genomes of mammals have been identified worldwide [24]. Previous studies showed that the dysregulation of lncRNAs may contribute to numerous human diseases in certain organs or tissues [25]. The lncRNA family is extremely large, and we still have only an incomplete understanding of the functions and regulatory mechanisms of the lncRNAs identified thus far. Few studies have examined the association between lncRNAs and PE, and these studies mainly focused on aberrant lncRNA expression and its effect on trophoblast cell function. In the present study, we found that the expression of MVIH was down-regulated in the placental tissues of PE compared with that in controls. Previous studies also showed that MVIH was up-regulated in hepatocellular carcinoma tissues and promoted tumour angiogenesis by affecting cell invasion and tube formation ability [19]. Based on the main mechanisms impeding uterine spiral artery remodelling in PE [26], we hypothesized that the low expression of MVIH is involved in the development of PE. However, it had remained unclear whether MVIH regulates the angiogenesis ability of trophoblast cells and vascular endothelial cells in the pathogenesis of PE.

To clarify the biological functions of MVIH in PE, we used loss- and gain-of-function assays to evaluate the ability of MVIH to stimulate angiogenesis in HTR-8/SVneo and HUVEC cells. The results demonstrated that silencing of MVIH inhibited the network formation ability of HTR-8/SVneo and HUVEC cells; the number of capillary-like networks was considerably decreased, and the expression of vascular endothelial growth factor, angiopoietin I and II was down-regulated in vitro. In contrast, the overexpression of MVIH increased the network formation ability and affected the expression of factors associated with angiogenesis correspondingly in trophoblast cells. Therefore, MVIH may promote angiogenesis, and low expression of MVIH may play an essential role in remodelling of the spiral arteries in PE.

We also performed MTT assays and transwell assays to further establish the various regulatory roles of MVIH in different trophoblast cells. The data revealed that knockdown of MVIH inhibited cell growth, migration and invasion. However, this knockdown was not proven to be associated with apoptosis. The results showed that the low expression of MVIH inhibits the proliferation, migration, invasion and angiogenesis of trophoblast cells, as well as affects the spiral artery remodelling process, which is involved in the pathogenesis of PE.

Given that proteins ultimately affect cell biological processes, we conducted mass spectrometry analysis to determine the expression of proteins regulated by MVIH. Considering the results, JunB was focused for further investigation for its modulation in angiogenesis and cell function.
Fig. 7 Jun-B involved in cell function phenotype. MTT assays and Transwell assays were used to determine the cell viability and migration for si-MVIH and pEGFP-Jun-B cotransfected HTR-8/SVneo cells, respectively (A–E). Network formation assays were performed to examine the network formation ability for si-MVIH and pEGFP-Jun-B cotransfected HUVEC cells, respectively (F–J). The expression of relevant vascular endothelial growth factor (VEGF) by qPCR after cotransfecting with effective siRNAs and pEGFP-Jun-B (K). (Values are mean ± SD; *: P < 0.05, **: P < 0.01)
proliferation. Jun-B, an important product of the Jun gene family, is a catalytically active endonuclease consisting of 299 amino acids. This protein forms two heterologous polymers with other proteins to form an essential transcription factor, activator protein-1 [27], which regulates cell proliferation and transformation and plays a regulatory role in the occurrence and metastasis of tumours [28]. It has been reported that Jun-B protein stimulates the growth of osteoblasts and chondrocytes by directly activating the transcription of cyclin A [29]. In terms of proliferation and angiogenesis, Jun-B protein can also reverse the proliferation and developmental defects of fibroblast cells induced by c-Jun deletion [30]. In this study, we demonstrated the effect of Jun-B on the proliferation and migration of trophoblast cells and the ability to undertake endothelial cell tube formation.

In summary, we found that MVIH is down-regulated in the placental tissues of PE and demonstrated that MVIH exerts vital effects on trophoblast cell invasion and angiogenesis. Taken together, our findings suggest that MVIH is associated with PE and is a candidate biomarker for the diagnosis and treatment of this condition. However, further studies using additional samples and analyses of the biological mechanisms involved are necessary.

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Conflicts of interest

No potential conflicts of interest were disclosed.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Sequence of primers and siRNAs.
Table S2 The detailed Mass Spectrometric database and protein identification.

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