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# MiR133b is involved in endogenous hydrogen sulfide suppression of sFlt-1 production in human placenta

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ABSTRACT

Increased production of soluble fms-like tyrosine kinase-1 (sFlt-1) from placenta is one of the major contributors to the development of preeclampsia. Our previous study has shown that hydrogen sulfide ($H_2S$) inhibits sFlt-1 release in placenta. In the present study, we sought to investigate whether endogenous $H_2S$ affects sFlt-1 production and elucidate which $H_2S$-producing enzyme is responsible for its effect in placenta. It was found that, besides cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), 3-mercaptopyruvatesulfurtransferase (3-MST) was identified in human placenta and mainly localized in syncytiotrophoblasts. There was no significant difference in expression level of 3-MST among preeclamptic and normal placentas. Treatment of cultured syncytiotrophoblasts with NaHS and L-cysteine suppressed sFlt-1 mRNA expression and caused a decrease in sFlt-1 protein content in culture media of the cells. Transfection of syncytiotrophoblasts with CBS siRNA and CSE siRNA reversed the above effects of L-cysteine. Furthermore, NaHS and L-cysteine treatment decreased the half-life of sFlt-1 mRNA and increased the expression of miR-133b targeting sFlt-1. MiR-133b expression was down-regulated in preeclamptic placentas and correlated with the level of CBS and CSE. These results indicate that $H_2S$ is an important regulatory factor in sFlt-1 production in placenta. Reduced $H_2S$ generation in placenta contributes to development of preeclampsia by enhancing sFlt-1 production.



## 1. Introduction

The vascular endothelial growth factor (VEGF) ligands/receptors play an essential role in both normal and pathological functioning of the endothelium [1]. Numerous studies has demonstrated that increased level of circulating soluble fms-like tyrosine kinase-1 (sFlt-1), the soluble secreted form of the vascular endothelial growth factor (VEGF) receptor, is one of the major contributors to the development of hypertension and proteinuria in preeclampsia since this factor acts as a VEGF and placental growth factor (PlGF) antagonist by making them unavailable for signaling to membrane bound receptors, thereby leading to dysfunction of endothelium [2–5]. The placenta is thought to be the main source of the approximate 20-fold increase in circulating sFlt-1 levels by the third trimester of normal pregnancy, compared with nonpregnant values [3–6]. Many studies have demonstrated that the production of sFlt-1 is significantly up-regulated in preeclamptic placentas, thereby leading to a marked increase in maternal circulating sFlt-1 [3,4,7,8]. Although it has been demonstrated that placental hypoxia and ischemia increase the production of sFlt-1 by the placenta [9,10], the mechanisms by which regulate sFlt-1 production in placenta remain elusive.

Hydrogen sulfide ($H_2S$), the third endogenous gaseous signaling transmitter in mammalian tissues, has recently been reported to be produced by placental tissues [11–13]. $H_2S$ synthesis from L-cysteine occurs naturally through the activity of the enzymes including cystathionine-γ-lyase (CSE, EC 4.4.1.1) and cystathionine-β-synthetase (CBS, EC 4.2.1.22) [14,15]. Recently, it has been

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demonstrated that 3-mercaptopyruvatesulfurtransferase (3-MST) in conjunction with cysteine (aspartate) aminotransferase (CAT) is responsible for generating $H_2S$ from L-cysteine in the brain and in the vascular endothelium [16]. $H_2S$ is implicated to be involved in many physiologic and pathologic processes including vasodilation [15], angiogenesis [17,18] and inflammation [19]. A number of studies have reported that CSE and CBS are expressed in human placentas and downregulated in preeclampsia [11–13]. Moreover, Wang et al. [20] have demonstrated that inhibition of CSE/$H_2S$ signaling results in features of preeclampsia including hypertension and increased circulating sFlt-1 level in pregnant mice, and decreasing CSE expression by CSE siRNA leads to an increase in sFlt-1 release in endothelial cells in placental explants from first trimester *in vitro*. More recently, we have shown that $H_2S$ donor and precursor suppress sFlt-1 release from human placenta [21]. However, which $H_2S$ generating enzyme is responsible for its effect and the mechanism underlying the effect of $H_2S$ remain to be elucidated. Although CSE and CBS have been identified in placenta, whether 3-MST is expressed in placenta has not been reported. In the present study, we firstly determined the localization of 3-MST in human placentas and the expression level of 3-MST in normal and preeclamptic placentas. We then studied the effects of $H_2S$ on sFlt-1 release and mRNA expression in cultured placental explants and cells and elucidate the underlying mechanisms.

## 2. Materials and methods

### 2.1. Tissue acquisition

The human placenta tissues were obtained from pregnant women with preeclampsia and healthy pregnant women who underwent elective cesarean section between 2010 and 2012 at Changhai Hospital, Shanghai, the affiliated hospital of Second Military Medical University (SMMU). Collections of tissues were performed with the approval of Specialty Committee on Ethics of Biomedicine Research, SMMU. Informed consent was obtained from all patients. Healthy women at term ($n = 23$) and women with preeclampsia ($n = 19$) were recruited in the present study. The information about the index of pregnancy and preeclampsia in these patients was reported previously [13]. All of the placenta samples were collected within 1 h of cesarean birth, and two small pieces of tissues from separate lobules were randomly taken in each placenta. The tissues were washed with normal saline, immediately frozen in liquid nitrogen and then stored at −80 °C.

### 2.2. Immunohistochemistry

Immunohistochemistry was performed as described previously [22]. Briefly, the sections were incubated with antibodies against CSE, CBS or 3-MST (at dilution 1:200–500) in PBS containing 1% BSA for 24 h at 4 °C. The bound antibodies were detected with the biotin–streptavidin–peroxidase system (UltraSensitive-SP-kit, MaiXin Biotechnology, Fuzhou, China) using diaminobenzidine (Sigma-Aldrich, St. Louis, MO) as chromogen. Counterstaining was performed with hemalum. Negative controls were performed by substituting primary antibody preabsorbed with a ten-fold excess of the blocking peptides. Two CSE antibodies from Santa Cruz (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and Abnova (Novus Biologicals, Cambridge,UK) and two antibodies against CBS from Santa Cruz and Sigma-Aldrich were used. 3-MST antibody was purchased from Santa Cruz.

### 2.3. Placental cell culture

Primary trophoblasts were cultured according to modified Kliman's method as described previously [23]. Briefly, villous tissue was minced and then digested with 0.125% trypsin (Invitrogen Corp., Carlsbad, CA) and 0.02% deoxyribonuclease-I (Sigma-Aldrich) in phenol red-free DMEM (Sigma-Aldrich). Cytotrophoblasts in dispersed cells were obtained through discontinued Percoll (Amersham Biosciences,Uppsala, Sweden) gradient (5–70%). Cells were then plated into twelve-well plates (Corning) at a density of $1.2 \times 10^6$/well and grown in phenol red-free DMEM with 10% fetal calf serum (FCS) at 37 °C in 5%$CO_2$-95% air. After 48 h-incubation, the medium was changed to FCS-free DMEM containing one of the following treatments: NaHS ($1–8 \times 10^{-5}$ M) and L-cysteine ($2.5–20 \times 10^{-4}$ M). Each treatment was performed in triplicate in each preparation of cells. The concentrations of above reagents were determined base on literature, our previous studies [13] and preliminary data. Cultured cells were fixed and immunostained for cytokeratin7 using primary antibodies (Santa Cruz) at a dilution 1:200 to assess cell purity. The results showed that placental cell cultures were predominantly cytokeratin7 positive (>95%).

### 2.4. Total RNA extraction and quantitative real-time RT-PCR

Total RNA extraction and quantitative real-time RT-PCR were carried out as described previously [13]. Briefly, total RNA was extracted by TRIzol reagent (Invitrogen) and then was reverse transcribed to generate cDNA by superscript reverse transcriptase (Invitrogen). Quantitative real-time PCR was carried out using MiniOpticon™ Real-Time PCR Detection System (BioRad, Hercules, CA). The reaction solution consisted of 2.0 μl diluted cDNA, 0.2 μM of each paired primer and 1 × PCR Master Mix (TaKaRa, Otsu, Japan). Amplification of the housekeeping genes β-actin and 18s-RNA was measured for each sample as an internal control for sample loading and normalization. The temperature range to detect the melting temperature of the PCR product was set from 60 to 95 °C. The comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used to determine the relative quantitation of gene expression for both target and housekeeping genes [24].

Total miRs of placental tissues and cells were extracted using miRcute miRNA Isolation Kit (TIANGEN, China) according to the manufacturer's instructions. The first strand cDNA synthesis of total miRNA was synthesized using miRcute miRNA First-Strand cDNA Synthesis Kit (TIANGEN, China). Quantification of miRs was carried out by miRcute miRNA qPCR Detection kit (TIANGEN, China). Amplification of the housekeeping gene U6 was measured for each sample as an internal control for sample loading and normalization. The temperature range to detect the melting temperature of the PCR product was set from 60 to 95 °C. The comparative Ct method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used to determine the relative quantitation of miR expression.

The primers which were used in the present study were illustrated in Table 1.

### 2.5. Western blotting analysis

Approximately 100 mg of tissues were homogenized in cold RIPA lysis buffer containing protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Cultured cells were scraped off the plate in the presence of the above buffer. 30 μg of protein samples were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were incubated with the antibody against CBS (Abcam), CSE (Abcam) or 3-MST (Santa Cruz) overnight at 4 °C, then washed and incubated with a secondary horseradish peroxidase-conjugated antibody (Santa Cruz). Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Santa Cruz). The chemiluminiscent signal was quantified by a GeneGnome HR