Elevated Expression of lncRNA *MEG3* Induces Endothelial Dysfunction on HUVECs of IVF Born Offspring via Epigenetic Regulation

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Research

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Abstract

Background: The cardiovascular dysfunction in children born after in vitro fertilization (IVF) has been of great concern, in our study, we aim to explore potential molecular mechanism for such long-term outcomes.

Methods Real-time qPCR was used to test long non-coding RNA *MEG3* and endothelium-derived factors, endothelial nitric oxide synthase (*eNOS*), endothelin-1(*ET1*), vascular endothelial growth factor (*VEGF*). Primary HUVEC after caesarean section was treated with different estradiol concentrations in vitro. Besides, knockdown of *MEG3* on HUVEC provided further evidence between *MEG3* expression and alteration of *NO, ET1, VEGF*. Then, by using pyrosequencing, we detected *MEG3* promoter methylation status.

Results: We found that the expression level of *MEG3* was higher in human umbilical vein endothelial cells (HUVECs) of IVF offspring than that in spontaneously born offspring. Furthermore, we found decreased expression of *eNOS, VEGF*, elevated expression of *ET1* in HUVECs from IVF offspring compared to spontaneously born offspring. We further confirmed the results from in-vivo experiments by demonstrating that high-estradiol intrauterine environments lead to abnormal expression of *MEG3* and endothelium-derived factors. Meanwhile, silencing *MEG3* expression decreased *ET1* expression, and increased nitrite, nitrate, *VEGF* secretion, which could correct the effect we observed in-vivo. With pyrosequencing technology, we found that elevated expression of *MEG3* in IVF offspring derived HUVECs was the result of hypomethylation of the *MEG3* promoter.

Conclusions: Our results demonstrated that higher expression of *MEG3* in IVF-born HUVECs, accompanied by lower secretion of *eNOS*, *VEGF*, and higher secretion of *ET1*, which is closely related with endothelial dysfunction, which together provide a potential mechanism addressing high-risk of hypertension in IVF offspring.

Background

Since the first in-vitro fertilization (IVF) pregnancy was reported in 1978 [1], an estimated seven million pregnancies have been achieved worldwide by IVF. Recently, increasing studies have proved that IVF-conceived fetuses are exposed to a high-estradiol environment in utero, which is closely correlated with increased risk of low birth weight (LBW) and small-for-gestational-age (SGA)[2, 3]. Disturbed intrauterine environment has been proven to be associated with rapid weight gain in early childhood, a higher risk of having high blood pressure in late childhood, a higher number of skin folds, and elevated fasting serum glucose levels concentrations[4, 5]. In our laboratory, we have also previously reported that, at the age of 3–13 years-old, the blood pressure of IVF-conceived Chinese children was higher than that of spontaneously conceived born children[3]. While recent studies have focused on the epidemiological consequences of IVF-conceived offspring, few have explored potential molecular mechanism for such outcomes.

“Fetal programming hypothesis” proposed by Barker et. al. suggested that the cardiovascular and related disorders derive from fetal adaptions in utero to maternal adverse environment which could permanently alters offspring’s postnatal metabolism and physiology[6]. For example, maternal undernutrition induced intrauterine growth retardation which caused a significant decrease in the number of nephrons in a year of birth, which might underlie the programming of hypertension[7, 8]. However, the question as to how intrauterine high-estradiol environment increases risk of hypertension in IVF offspring later in life remains contentious[9].

Epigenetic modifications, such as DNA methylation, histone modifications, and non-coding RNAs are involved in mediating how early life impacts later health. Long non-coding RNAs (lncRNAs) are non-coding transcripts that are longer than 200 nucleotides in length. So far, abundant studies have demonstrated that dysfunction of lncRNAs are associated with the pathogenesis and progression of a broad range of diseases, including cardiovascular disease[10]; examples of this include *CDKN2B* antisense RNA 1 (*ANRIL*), which has been implicated in atherosclerosis [11], and metastasis associated lung adenocarcinoma transcript 1(*MALAT1*), which has been shown to stimulate vascular growth in vivo and drive the proliferation of a migratory endothelial cells in vitro[12]. Maternally expressed gene 3 (*MEG3*) is a long non-coding RNAs located at chromosome 14q32.3 in humans. *MEG3* is expressed in many normal tissues, and lost in several human tumors and tumor cell lines[13]. *MEG3* is also expressed in arterial endothelial cells [12], and *MEG3* knock-out enhances the expression of *VEGF* signaling pathway genes in the brain[14]. Notably, *MEG3* is the only significantly increased lncRNA in senescent HUVEC, which suggests that *MEG3* may mediate endothelial dysfunction in aging[15]. Hypoxia condition leads *MEG3* expression significantly upregulated, accompanied by endothelial cells proliferation, migration and angiogenesis, as well as cell death and growth arrest [16].

In this study, we investigated the role of lncRNA *MEG3* in umbilical cord blood vessels of IVF born offspring, which might offer a potential mechanism for adult chronic cardiovascular diseases of fetal origin.

Materials And Methods

Study Population

We reviewed the records of 421 singletons naturally born and 482 singletons born by IVF-ET in our hospital during 2013–2016. We evaluated the fetal growth measurement and ratio of umbilical cord end systolic peak over end diastolic peak (S/D). Furthermore, we recruited 21 singletons born by fresh IVF-ET and 22 singletons born by naturally conceived (NC) from Jan.1, 2016- Jan.1, 2017 for evaluation of the function of human umbilical endothelial cells. Baselines of parental characteristics were collected in the third trimester; these included maternal blood pressure, heart rate, the serum levels of fasting blood glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, homocysteine, and serum estradiol concentration.

Children born with cardiovascular diseases, congenital anomalies, premature delivery, small for gestational age were excluded. We also excluded children whose mothers had gestational complications, such as gestational diabetes, preeclampsia, and others.

Tissue Samples

Umbilical vessels and cord blood were collected from twenty-one IVF and twenty-two NC singleton pregnancies immediately after cesarean delivery in the Women's Hospital. The detailed characteristics of these samples are listed in Supplementary Table 1. Mothers with previous cardiovascular diseases, or other gestational complications were also excluded. Mothers of IVF babies had normal ovarian function and experienced controlled ovarian hyper-stimulation with gonadotropins followed by the standard luteal long gonadotropin-releasing hormone agonist down-regulation protocol for the first IVF cycle. Embryo transfer was performed after 2-3 days of egg retrieval. All babies included met the following criteria: maternal age between 25 and 35 years old; full-term delivery; singleton pregnancy; child's birth weight between 2500-4000 g; no indication of pregnancy complication; no birth defects; and no cardiovascular diseases. We examined the E2 levels in cord blood from newborns with the E2 kit (H-Estradiol E2, Abcam, ab108640).

Isolation and culture of Primary human umbilical Vein Endothelial Cells

The protocols for HUVEC isolation and culture were performed and modified according to Crampton et. al. [17]. Briefly, the fresh vein was filled with a solution containing 1mg/ml collagenase, the cord was incubated in pre-warmed phosphate-buffered saline at 37 ℃ for 30 minutes, and cells were cultured in 5% fetal bovine serum combined with endothelial cell medium (ECM, ScienCell, cat. #1001). The HUVECs were used between passage 3 to 5.

Small interfering RNA-mediated MEG3 knock-down

SiRNA oligonucleotides were purchased from Thermo Fisher Scientific. The sequences of siRNAs targeting Meg3 are as follows (5'–3'): sense, GCUCAUACUUUGACUCUAUTT; anti-sense, AUAGAGUCAAAGUAUGAGCTT. The sequences of negative control (NC) are as follows (5'–3'): sense, UUCUCCGAACGUGUCACGUdTdT; anti-sense, ACGUGACACGUUCGGAGAAdTdT. The siRNAs against Meg3 were reverse-transfected into cells at a dose of 10nm in 6-wells plates using the Lipofectamine 3000 reagent (ThermoFisher, Catalog. L3000008) for 48h.

Quantitative Realtime PCR Analysis

Total RNA was extracted from the tissue sample and primary human umbilical vessel cells using the TRIzol Reagents (Invitrogen Life Technologies, Carlsbad, CA, USA). cDNA was synthesized using PrimerScript RT Reagent Kit (Takara, RR037A, Japan) in a 20 μl reaction containing 0.5-1ug of total RNA. Realtime quantitative PCR was performed using ABI Prism 7900HT (Applied Biosystem, Foster City, CA). GAPDH was the internal control. Full list of primer sequences is shown in Supplementary Table 2.

Western Blot

The protein was extracted from HUVECs tissues with lysis buffer, which was separated using 10% SDS-PAGE. Western Blots was performed using polyvinylidene fluoride membrane and the antibodies for DNMT3A (Cell Signaling, 32578, used at a dilution of 1:1000), DNMT3B (Cell Signaling, 57868, used at a dilution of 1:1000), beta-ACTIN (Abcam, ab8226, used at a dilution of 1:5000). Protein bands were visualized by the enhanced chemiluminescence system (Pierce, Rockford, IL).

ELISA assay

Umbilical cord blood and cell supernatants were collected after cesarean delivery. ELISA kits were used for determination of NO (Nitric Oxide Assay, ab65328, abcam, UK), VEGF (H-VEGF, DVE00, R&D, USA), and ET-1 (H-Endothelin-1, QET00B, R&D, USA) levels. The procedures were performed according to the manufacturers' protocols. For the cell-line culture, three replicates of data were used for statistical analyses.

DNA isolation and bisulfite conversion

Total genomic DNA was isolated from umbilical vessel tissues using Genomic DNA Purification Kit (Invitrogen, cat. K0512, USA). Bisulfite was converted using the EpiTect bisulfite kit (Qiagen, Valencia, CA) according to the manufacturerer’s instructions to deaminate cytosine to uracil; 5-methyl-cytosine was protected from deamination. PCRs were performed in a ABI 9700 PCR System (Applied Biosystems, USA) using an annealing temperature of 56°C.

DNA Methylation analysis by pyrosequencing

The bisulfite converted DNA was amplified using Hotstart Plus DNA polymerase (Qiagen). PCR products were immobilized on streptavidin-sepharose beads (GE Healthcare), washed, denatured and released into annealing buffer containing sequencing primer which is described in Supplementary Table 3. Pyrosequencing was carried out on a PyroMark Q96 instrument (Qiagen) according to the manufacturer's instruction. % methylation was calculated using the Pyro Q CpG software (Qiagen).

Statistical Analysis

Data were analyzed using SPSS 18.0, and were presented as mean ± SD or mean ± SE. Statistical analysis including unpaired two-tailed Student’s t-test was performed as described in the figure legends or Excel legends. $P < 0.05$, $P<0.01$, or $P<0.001$ was considered statistically significant.

Results

Retrospective analysis

Baseline and perinatal characteristics of babies who were born in the past 4 years (2013–2016) in our hospital are shown in Table 1. We found that random maternal blood sugar and Triglycerides in IVF

mothers were slightly higher than in NC mothers (4.94 ± 1.32 mmol/L VS 4.761 ± 1.23 mmol/L, P < 0.05; 3.73 ± 1.98 mmol/L VS 3.06 ± 1.84 mmol/L, P < 0.0001), while high density lipoprotein (HDL) cholesterol was significantly lower in IVF mothers (1.74 ± 0.44 mmol/L VS 1.81 ± 0.40, P < 0.05). The results might be related with higher circulating estrogen concentration during pregnancy, which causes dysfunction of carbohydrate and lipid metabolism, including glucose intolerance, insulin resistance, increasing very low-density lipoprotein (VLDL) production, and other effects [14, 18, 19]. Notably, in terms of fetal development, there are no significant difference in birth weight and S/D ratio, which is considered to relate to umbilical cord vessel function, while Bi-parietal and femur diameter were smaller in IVF born babies (9.16 ± 0.44 VS 9.26 ± 0.41, P < 0.01; 6.88 ± 0.44 VS 7.02 ± 0.41, P < 0.0001).

Table 1
The characteristic of children at birth, fetal development index (36–38 weeks ultrasound) and the maternal characteristics at conception.

Characteristics	NC (N = 421)	IVF(N = 482)	P-value
Maternal age, yr	30.49 ± 3.69	30.85 ± 3.95	0.34
BMI before delivery, kg/m2	22.56 ± 2.25	25.32 ± 3.69	0.23
Gestational age, wk	38.44 ± 1.84	37.48 ± 2.41	< 0.0001
Systolic BP, mmHg	119.7 ± 11.90	120.9 ± 14.7	0.16
Diastolic BP, mmHg	74.49 ± 7.38	75.57 ± 9.44	0.06
Heart rate, bpm	86.29 ± 8.40	87.4 ± 9.00	0.06
random blood sugar, mmol/L	4.761 ± 1.23	4.94 ± 1.32	< 0.05(0.04)
Triglycerides, mmol/L	3.06 ± 1.84	3.73 ± 1.98	< 0.0001
Total cholesterol, mmol/L	6.19 ± 1.23	6.28 ± 1.31	0.27
HDL cholesterol, mmol/L	1.81 ± 0.40	1.74 ± 0.44	< 0.05(0.01)
LDL cholesterol, mmol/L	2.95 ± 0.82	2.82 ± 0.88	0.06
Male birth, n, %	52.0%±2.4%	49.6%±2.2%	0.49
Birth weight, g	3250 ± 478.1	3183 ± 643.3	0.08
Bi-parietal diameter, cm	9.26 ± 0.41	9.16 ± 0.44	< 0.01(0.003)
Femur diameter, cm	7.02 ± 0.41	6.88 ± 0.44	< 0.0001
S/D ratio	2.174 ± 0.41	2.191 ± 0.44	0.50

Data are presented as Mean ± SD, NC: naturally conceived. IVF: in vitro fertilization. Women with Pregnancy complications including diabetes mellitus, arterial hypertension and dyslipidemia were excluded.

Baseline and perinatal characteristics

Baseline and perinatal characteristics of the study population are shown in Supplemental Table 1. The maternal characteristics and delivery data were comparable with the NC group, except levels LDL cholesterol, which is slightly elevated in IVF mothers (3.06 ± 0.73 VS 2.47 ± 0.66, P < 0.01). The serum estradiol concentration from umbilical cord blood vessels in IVF women was significantly higher than that in NC women, which is coincidence with our previous results[3]. However, there were no obvious differences in fetal birth weight or male birth rate between the NC and IVF born offspring.

Up-regulated expression of MEG3 in IVF HUVEC

In order to identify the mRNA expression level of *MEG3* in HUVEC, quantitative real-time polymerase chain reaction (qPCR) was performed and we found that the expression level of *MEG3* was significantly higher in IVF born HUVECs (Fig. 1A), which suggests that *MEG3* could play a role in HUVEC development and function.

Endothelium-derived factors altered in HUVEC of IVF offspring

We previously found that offspring born after IVF have a high blood pressure during age 3–13 years-old [3], so does offspring around 2 years-old recruited in our study (85.12 ± 5.23 VS 82.01 ± 4.85, P < 0.05) (Supplementary Table 1). Therefore, we focused on the endothelium-derived factors, such as endothelial nitric oxide synthase (*eNOS*), which is classical vasodilation factor, endothelin-1(*ET1*), one of the critical vasoconstriction factors, and vascular endothelial growth factor (*VEGF*), which is related with endothelial cell proliferation, angiogenesis, and vascular permeability. qPCR showed that *eNOS* and *VEGF* expression were significantly decreased in IVF offspring group (Fig. 1B, 1D), while *ET1* expression was significantly increased (Fig. 1C). Since NO has a very short half-life of several seconds, we tested the levels of the first and second oxidation products of NO, nitrite and nitrate, respectively, using ELISA. The results showed that nitrite concentration was significantly lower in the umbilical cord serum of IVF offspring (Fig. 1F), while there was no difference in nitrate concentration (Fig. 1E). Furthermore, *ET1* was significantly higher in IVF offspring (Fig. 1H) while *VEGF* secretion was decreased (Fig. 1G), which is coincidence with the mRNA level. These results suggested that intrauterine high-estradiol environment might lead to endothelial dysfunction associated with vascular activity, since HUVECs share a common embryological origin with other fetal vessels.

Knockdown of MEG3 in HUVEC decreases ET1 expression and increases secretion of nitrite and VEGF in vitro

Katharina M. Michalik et. al. reported that *MEG3* was highly rich in the nuclear fraction, and profoundly increased by hypoxia[12]. Given this data, we further explored the function of *MEG3* in endothelial cells by silencing *MEG3* expression with one specific siRNA, which could reduce endogenously expressed *MEG3* by 85% (Fig. 2A). Through silencing *MEG3*, we discovered that *MEG3* reduction significantly suppressed *ET1* mRNA levels and *VEGF* mRNA levels (Fig. 2C, 2D), but would not arouse any significant changed in

eNOS expression (Fig. 2B). Through ELISA, we demonstrated that nitrate and nitrate secretion was significantly increased with *MEG3* suppression (Fig. 2E, 2F), while *ET1* secretion was decreased (Fig. 2H). We also observed no significant difference in *VEGF* secretion with *MEG3* suppression (Fig. 2G)

High expression level of MEG3 in human umbilical vein endothelial cells was controlled by DNA methylation

As a maternal imprinted gene, *MEG3* expression is regulated by two critical different methylation regions (DMR): intergenic region between *DLK1* and *MEG3* (IG-DMR), and MEG3 DMR(CG7)[20] (Fig. 3A). Pyrosequencing showed no difference in the average methylation status in IG-DMR between IVF offspring and spontaneously born offspring (Fig. 3D), while 5 sites are in hypermethylation status from the aspect of single CpG status in the IG-DMR (Fig. 3B). However, MEG3-DMR, which is located in the − 287 to -120 region of the human *MEG3* promoter (which has eight differentially methylated CpGs), showed significantly reduced methylation status in IVF HUVECs, which consisted 6 sites hypomethylation. (Fig. 3C, 3E).

Three DNMTs determine the methylation status of DNA methylation in humans: DNMT3a and DNMT3b are responsible for de novo methylation, while DNMT1 is conservatively expressed and required for maintenance of methylation[21]. We performed qPCR to detect the expression of DNMTs, and the results indicated that there was a significant reduction of DNMT3A and DNMT3B in IVF HUVECs compared with spontaneous-born HUVECs (Fig. 3F, 3G) both in mRNA level and protein levels, which was consistent with the DNA methylation status of *MEG3* DMR.

Effects of high estradiol concentration on primary human umbilical vein cells

In order to understand the cause of the up-regulation of *MEG3*, we cultured primary HUVECs in three different estradiol concentrations (10^{-10} mmol/L, 10^{-8} mmol/L, 10^{-6} mmol/L). We found that as estradiol concentration went up, *MEG3* and *ET1* expression were significantly increased (Fig. 4A, 4D), *VEGF* expression decreased (Fig. 4C), which were in accordance with the in vivo results. However, there was no significant difference of *eNOS* expression when treated with different estradiol concentration (Fig. 4B).

Discussion

In this study, we demonstrate for the first time that long coding RNA *MEG3* suppresses vasodilation while promoting vasoconstriction in HUVECs of intrauterine high-estradiol environment. Our molecular findings were consistent with follow-up data showing that IVF offspring have a higher incidence of hypertension. Meanwhile, these alterations may originate from regulation of MEG3-DMR at the epigenetic level, leading to higher expression of *MEG3*. This study may provide a novel mechanism and potential theory for fetal-origin adult diseases originated from high-estradiol intrauterine environment.

As IVF births continue to increase in popularity, it is important to consider the health effects on the offspring. Several studies have shown that short-term outcome of IVF-born infants are positive [22]; however, mounting evidence suggests an association between IVF-born infants and long term health and developmental consequences, particularly the early-onset of cardiovascular diseases [23], such as hypertension.

Current evidence suggests that the long-term health consequences in IVF-born offspring may be related to low birth weight (LBW) [24]. In our study, IVF born babies had slightly lower birth weights compared to spontaneously conceived babies, although there was no statistical significance. The potential reason for LBW in IVF-born babies is chronic placental dysfunction[25], such as increased apoptosis of trophoblast cells due to a high estradiol concentration in utero[26].

Endothelium is one of the largest organ systems by surface area. Normal endothelial function is critical in keeping the symbiotic balance between vasoconstrictive (namely like *ET1*) and vasodilatory (namely via NO) stimuli, thus a disturbance in normal endothelial function is a predictor of future adverse cardiovascular events[27]. Since endothelial cells from the cord may reflect characteristics of the offspring's vascular system[28], in our study, we sought to investigate the function of HUVECs derived from IVF born babies. As we know, the endothelial-dependent response to vasodilate is fundamentally regulated by a release of nitric oxide (NO) synthesized from the amino-acid L-arginine by endothelial nitric oxidase synthase (*eNOS*)[29]. In addition to NO as one critical factor in endothelial function, enhanced activity and levels of *ET1* also are related to endothelial dysfunction, by stimulating NADPH oxidase-derived ROS production which inhibits NO-mediated endothelial relaxation and mediating ET_A receptors to blunt NO relaxant responses[30]. Vascular endothelial growth factor (*VEGF*) plays a central role in endothelial function, including stimulating endothelial proliferation, migration, and nitric oxide release; thus, *VEGF* might exhibit the same tendency with NO[31]. In our study, we found that the IVF group has lower *eNOS* and *VEGF* expression, lower secretion of nitrate, nitrite, *VEGF* concentration, and higher *ET1* expression and production, all of which might lead to endothelial dysfunction in IVF born babies. Although certain concentration and time course exposures of estradiol may improve NO product and thus enhance vasodilation[32], as observed in our study, it may be related to extremely elevated concentrations of estradiol in vitro and long-term estradiol exposure in vivo for IVF born babies, which may result in endothelial wall damage.

The recent identification of a novel group of mediators known as long-coding RNAs (lncRNAs) has provided a large quantity of new biology to explore for cardiovascular risk reduction. Several lncRNAs take part in acute myocardial infarction (eg, Novlnc6) and heart failure (eg, *Mhrt*), control hypertrophy and apoptosis of cardiomyocytes [33], and regulate vessel growth and function in the vascular system, such as *MALAT1*[12]. *MEG3* is an imprinted gene belonging to the imprinted *DLK1-MEG3* locus at chromosome 14q32.3 in humans. The gene expression in this locus is tightly controlled by at least two differentially methylation regions (DMRs): the intergenic DMR (IG-DMR) and the MEG3-DMR. Numerous studies have implicated the involvement in *MEG3* in a myriad of biological processes, notably as a tumor suppressor[13, 34]. *MEG3* is also involved in many cardiovascular functions, including angiogenesis

through *VEGFA* and *VEGF1R* expression[35, 36] and smooth muscle cell proliferation through the p53 pathway. In this study, we show that HUVECs from IVF born offspring have high expression levels of *MEG3*. Taken together, these results suggest that *MEG3* may be involved in the etiology of cardiovascular diseases of offspring born in intrauterine high-estradiol environment.

Conclusion

Altogether these data show that IVF neonates have an abnormal endothelium response in human umbilical vein endothelial cells, decreased *eNOS* expression and synthesis of NO in endothelial cells, and increased *ET1* expression and secretion in umbilical cord serum, which may be the result of elevated expression of *MEG3*. These data suggest that the abnormal expression of *MEG3* may contribute to the development of cardiovascular disease of offspring born in intrauterine high-estradiol environment later in life.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Research and Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University, China. All participants signed informed consents. We design our study in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

Y. J., H. Z. and H. C. contributed to collection, analysis, and interpretation of data as well as manuscript preparation. M. M. Y. and Y. C. Y. contributed to data collection and analysis. S. N. H., Y. T. X. and F. L. contributed to interpretation of data. M. S. contributed to edit the language. Q. L. and Y.M.Z. contributed to study design and data interpretation and the manuscript preparation. Q. L. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figures

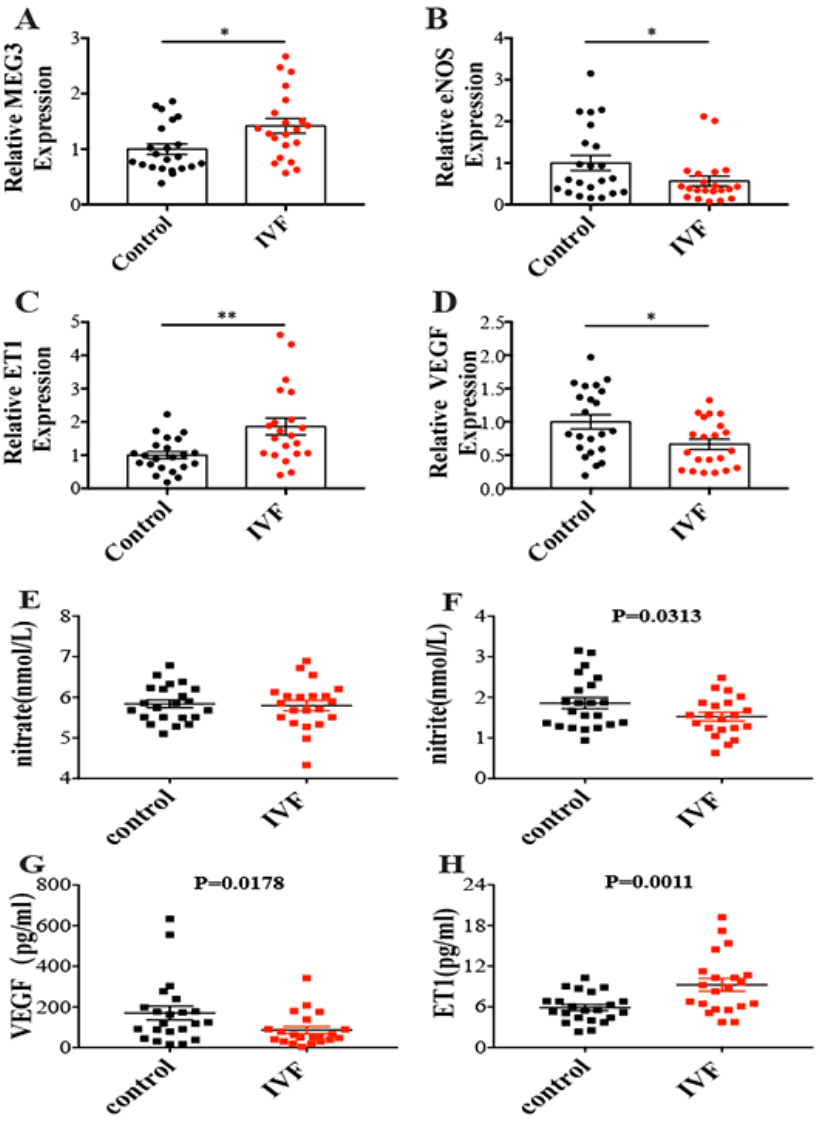

Figure 1 the mRNA expressions of MEG3 and endothelium-derived genes and serum secretions related with endothelial function. (A), (B), (C), (D) RNA levels determined by RT-qPCR. Data were analyzed with the Eq. $2^{-\triangle\triangle CT}$, where $\triangle\triangle CT = \triangle CT$ (treatment group) $- \triangle CT$ (control group), and $\triangle CT =$ CT (sample) - CT (internal control). The values were normalized to GAPDH mRNA levels. (E), (F), (G), (H) ELISA was performed to detect the secretion of nitrate(E), nitrite(F), VEGF (G) and ET1 (H). Control has 22 samples and IVF group has 21 samples. In all panels, data are presented as mean ± SE, *P<0.05, **P<0.01. Significance was determined by Student t test.

Figure 1

Figure 1

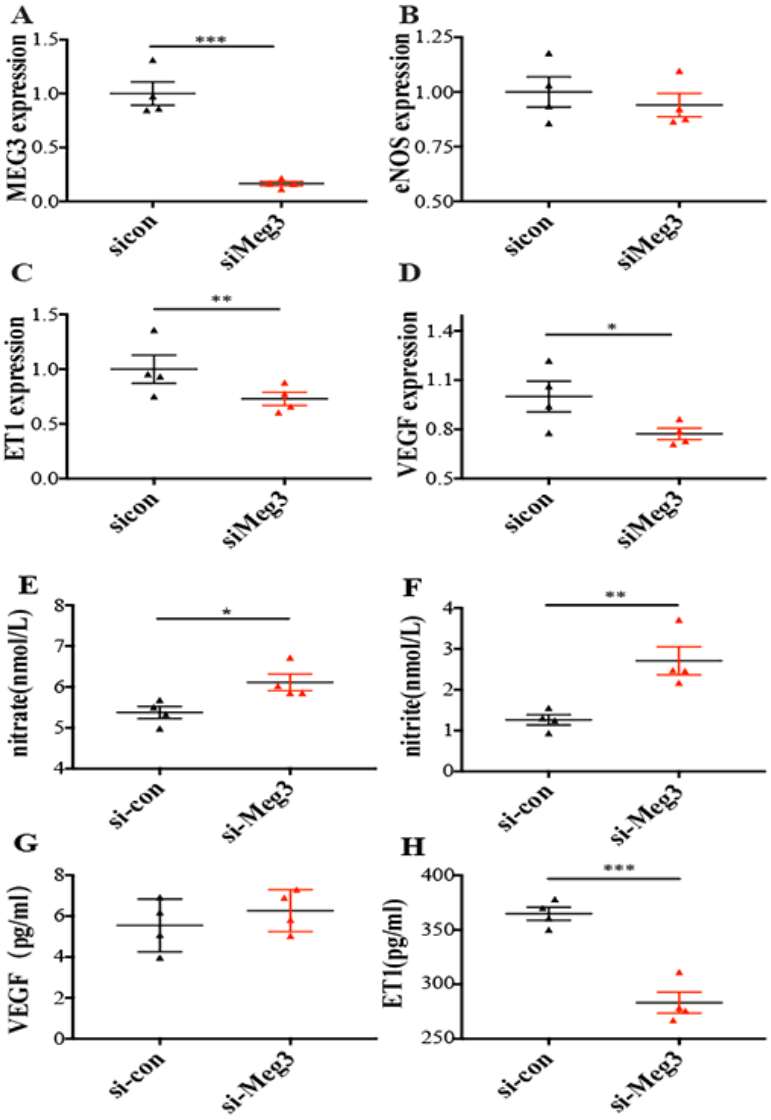

Figure 2 the mRNA expression of endothelium-derived genes in MEG3 knockdown treatment in-vitro primary human umbilical endothelial cells and serum secretions of these target proteins in supernatant liquids. (A), (B), (C), (D) RNA levels determined by RT-qPCR. Data were analyzed with the Eq. $2^{-\triangle\triangle CT}$, where $\triangle\triangle CT = \triangle CT$ (treatment group) $- \triangle CT$ (control group), and $\triangle CT = \triangle CT$ (sample) $- \triangle CT$ (internal control). The values were normalized to GAPDH mRNA levels. (E), (F), (G), (H) ELISA was performed to detect the secretion of nitrate(E), nitrite(F), VEGF (G) and ET1 (H). Both control and IVF group have 4 samples and tried for triple times. In all panels, data are presented as mean ± SE, *P<0.05, **P<0.01, ***p<0.001. Significance was determined by Student t test.

Figure 2

Figure 2

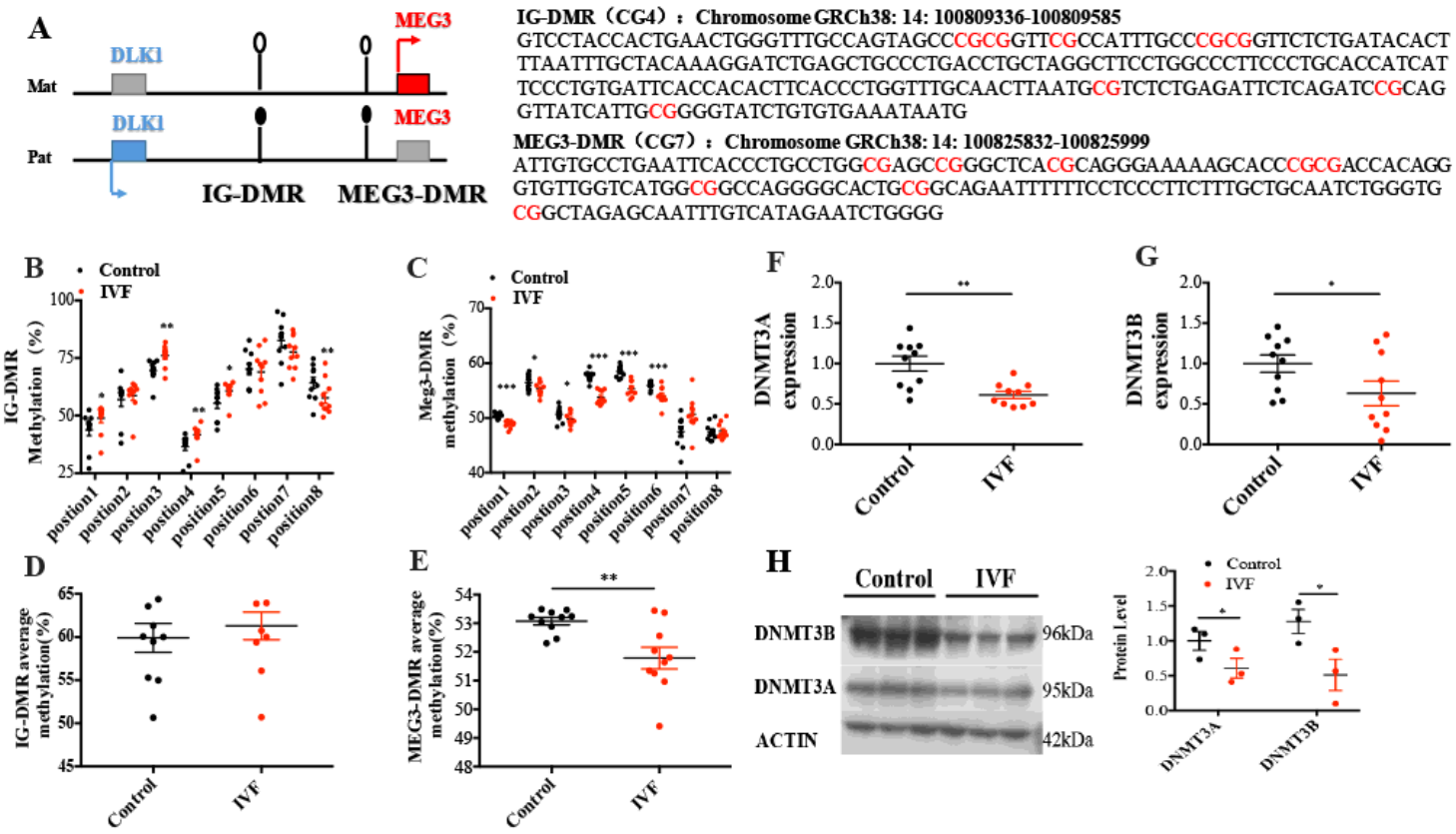

Figure3 Methylation analysis of IG-DMR/MEG3-DMR by pyrosequencing. (A) Schematic representation of human imprinted locus, showing the relative position of the DLK1 and MEG3 genes and indicating the location of the two DMRs known to contribute to MEG3 imprinting. Exons are known as blue (DLK1 gene) and red (MEG3 gene) rectangles with arrows for transcription start sites. Under the genetic map listed specific gene sequence of the two DMRs. (B) Methylation status of individual DNA strands of IG-DMR containing 8 CpG sites and the average methylation ratio in each CpG site; (C) Methylation status of individual DNA strands of MEG3-DMR containing 8 CpG sites and the average methylation ratio in each CpG site. (D), (E) the average methylation status of IG-DMR and MEG3-DMR, ten patients were included in each CpG site. (F)(G) Dnmt3A, Dnmt3B RNA levels determined by RT-qPCR. (H) Dnmt3A, Dnmt3B protein levels determined by Western-Blot. Data were analyzed with the Eq. $2^{-\triangle\triangle CT}$, where $\triangle\triangle CT = \triangle CT$ (treatment group) - $\triangle CT$ (control group), and $\triangle CT = \triangle CT$ (sample) - $\triangle CT$ (internal control). The values were normalized to GAPDH mRNA levels. For western blot, the values were normalized to β-ACTIN protein levels. For pyrosequencing and RT-qPCR, there were 10 samples both for control and IVF group, for Western-blot, there were 3 samples both for control and IVF group. In all panels, data are presented as mean ± SE, *P<0.05, **P<0.01, ***P<0.001. Significance was determined by Student t test.

Figure 3

Figure 3

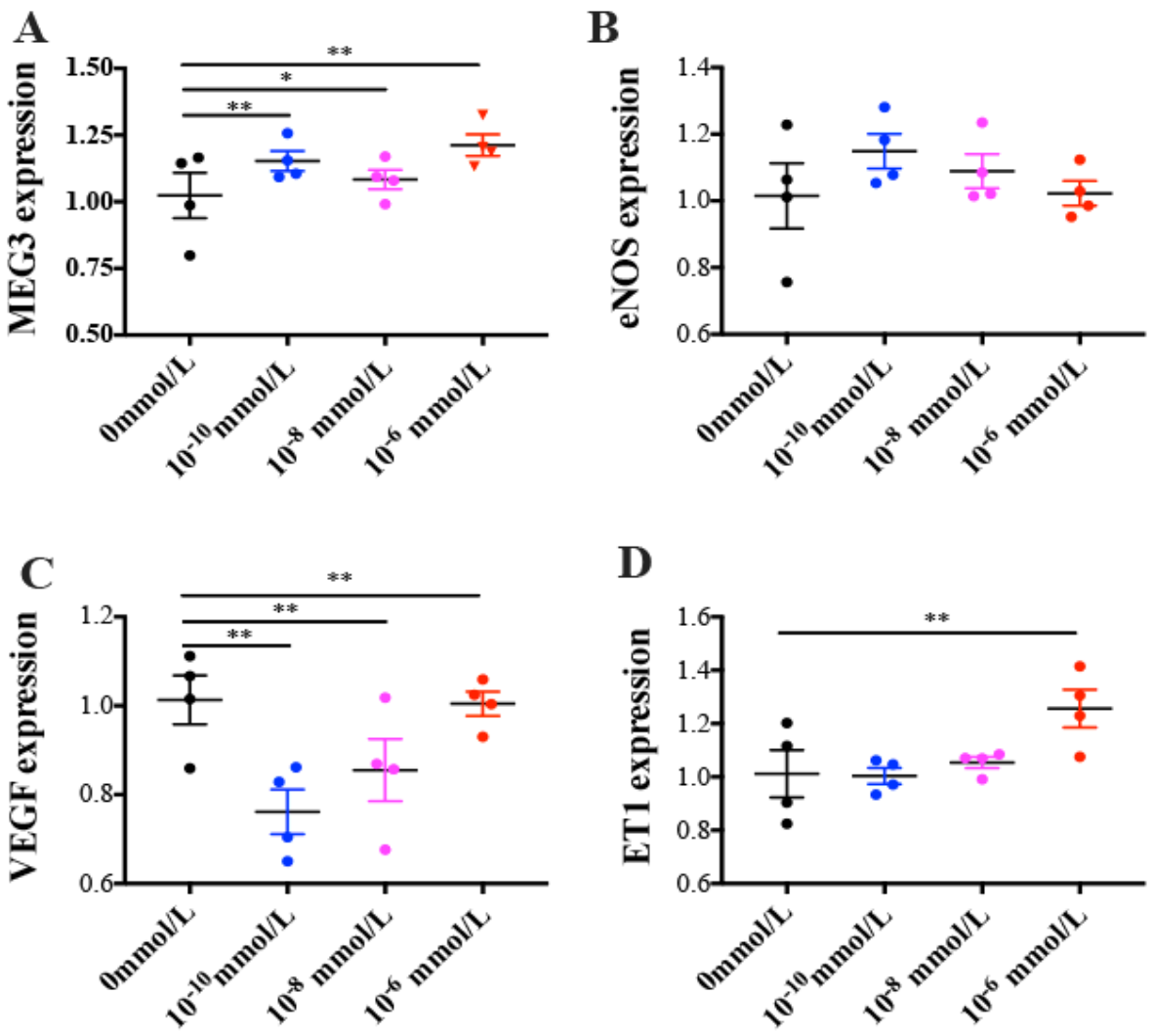

Figure 4 Effects of different estradiol concentration on primary human umbilical vein endothelial cells. (A), (B), (C), (D) represented MEG3, eNOS, VEGF, and ET1 RNA levels which were determined by RT-qPCR. The values were normalized to GAPDH mRNA levels. For different estradiol treatment group, there were 4 samples and tried for triple times. In all panels, data are presented as mean ± SE, *P<0.05, **P<0.01. Significance was determined by Student t test.

Figure 4

Figure 4

Supplementary Files

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