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Methylation-reprogrammed AGTR1 results in increased vasoconstriction by angiotensin II in human umbilical cord vessel following in vitro fertilization-embryo transfer

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ABSTRACT

Aims: Assisted reproductive technologies (ART) have been widely used to treat infertility, which may impact on fetuses and offspring. This study investigated the effects of in vitro fertilization-embryo transfer (IVF-ET) on angiotensin II (AII)-mediated vasoconstrictions in umbilical cord vein, and explored possible reprogrammed methylation mechanism.

Materials and methods: Human umbilical cords were randomly divided into ordinary pregnancy and IVF-ET pregnancy. Vascular studies with AII as well as its specific receptor antagonists losartan and PD123,319 were conducted. Real-time quantitative PCR, Western blotting, and methylation analysis by bisulfite sequencing were performed with the cord vessel samples.

Key findings: In IVF-ET group, the maximal response to AII in umbilical vessels was significantly greater than that in the ordinary pregnancy. Using losartan and PD123,319, angiotensin receptor subtype 1 (AT1R) was found mainly responsible for the enhanced contraction in the umbilical vein of IVF-ET pregnancy. Decreased mRNA expression of DNMT3A was found in umbilical vein of IVF-ET group. Hypomethylation of the AGTR1 gene (gene encoding AT1R) in the umbilical veins of the IVF group was found. The data suggested that the IVF-ET treatments altered AII-mediated vasoconstrictions in umbilical veins, which could be partially attributed to the increased expression of AT1R.

Significance: The hypo-methylation of the AGTR1 gene caused by IVF-ET might play important roles in altered vasoconstrictions, impacting on cardiovascular systems in the long run.

1. Introduction

Assisted reproductive technologies (ART) have become a common method of infertility treatment since 1978, when Dr. Robert Edwards and Dr. Patrick Steptoe have developed the first in vitro fertilization and embryo transfer (IVF-ET) baby [1]. Nearly 10%–15% couples in the world have difficulty in conceiving children and seek ART at least once during lifetime [2]. There were about 8 million in vitro fertilization babies born during the past 40 years. China has become the country with the most birth of IVF babies, approximately 200 thousand each year [3].

The processes of IVF is involved in stimulating the ovaries, retrieving multiple eggs via a trans-vaginal ultrasound-guided needle, retrieving the sperm sample, fertilization, and embryo culture [4]. If all those procedures go well, some of the retrieved eggs will be fertilized by the sperm cells and then, become embryos. In the vast majority of the cases one or two of those healthy embryos will be transferred to females' uterus. The rest of the embryos could be cryopreserved for the purpose of using in future. The probability of successful conception for the healthy women aged 23–34 years old is about 25% in one reproductive cycle. Since the success of the IVF-ET, more techniques, including gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), intracytoplasmic sperm injection (ICSI), preimplantation genetic screening (PGS) and preimplantation genetic diagnosis (PGD) have been developed. It is known that early stages of embryonic development are very sensitive to their micro environments, therefore, short and long term effects or influence of the tedious ART procedures becomes a matter of concern, especially for perinatal and long term

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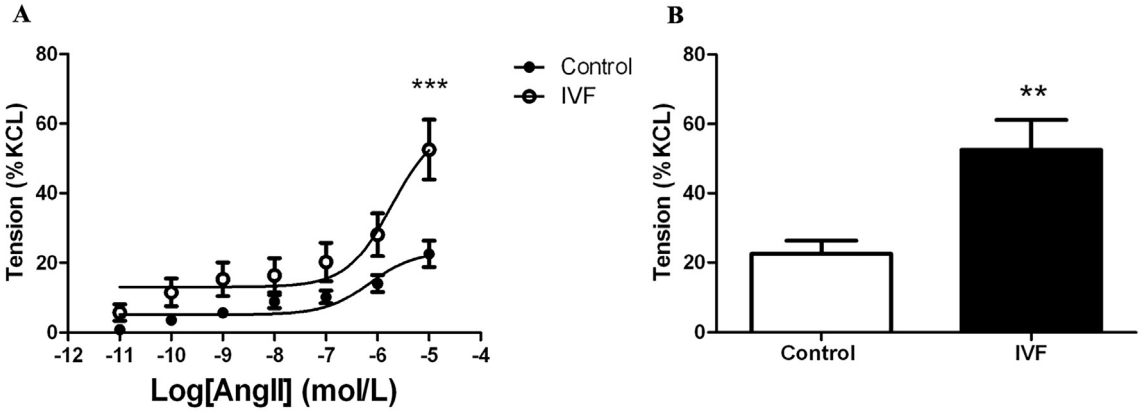


Fig. 1. (A) Angiotensin II induced concentration-dependent vasoconstrictions in HUV of the ordinary and IVF-ET pregnancy. (B) Angiotensin II at a dose of 10^{-5} induced maximal contractions in ordinary and IVF-ET pregnancy. The maximal response to AII at a dose of 10^{-5} in IVF-ET group was significantly greater than that in the control. (***, $p < 0.001$, two-way ANOVA followed by Bonferroni post hoc tests, **, $p = 0.002$, two-tailed unpaired Student's *t*-test, n = 30. Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.)

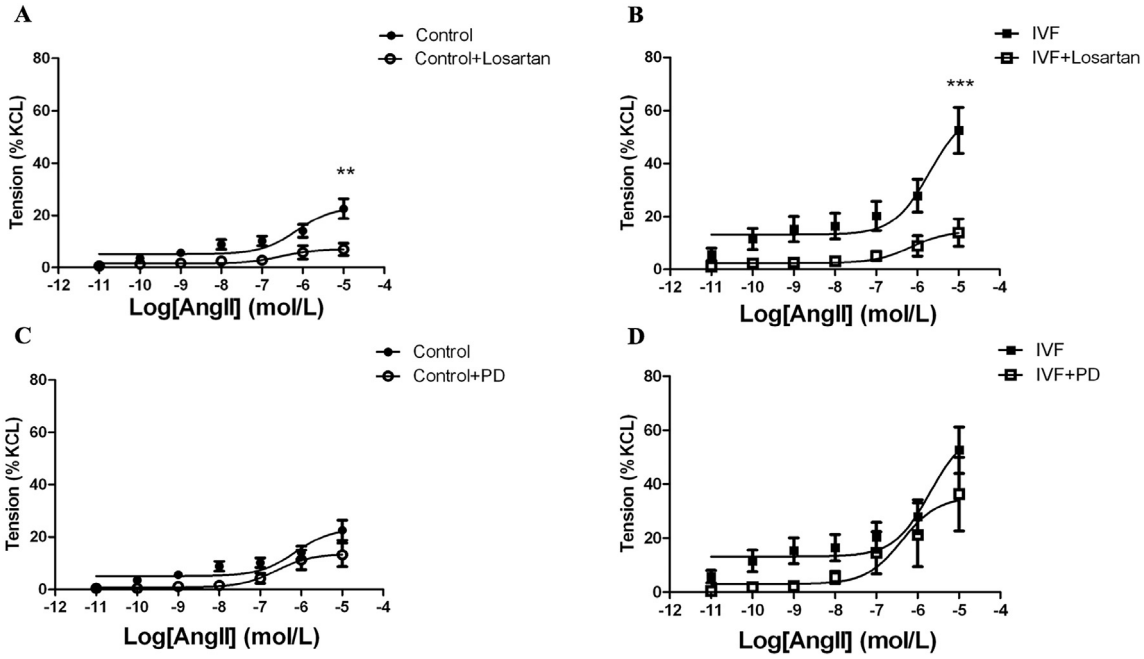


Fig. 2. (A) Response curves of AII-induced contractions with/without losartan in ordinary pregnancy. (B) Response curves of AII-induced contractions with/without losartan in IVF-ET pregnancy. After being incubated by losartan, the contractions of the umbilical cord veins induced by AII are mostly blocked. (C) Response curves of AII induced contractions with/without PD123,319 in ordinary pregnancy. (D) Response curves of AII induced contractions with/without PD123,319 in IVF-ET pregnancy. The PD123,319 showed no effect on the AII induced contractions of umbilical cord veins. (Losartan, an AT1R antagonist; PD123,319, an AT2R antagonist; AII, angiotensin II; **, $P < 0.01$; ***, $P < 0.001$, two-way ANOVA followed by Bonferroni post hoc tests, n = 28. Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.)

heath of ART babies.

Despite several studies showed benefit effects of ART for pregnancy [5,6], quite a few studies suggested that the IVF babies showed a significant increase in perinatal mortality [7–9]. The IVF-ET singletons are more likely to face adverse perinatal outcomes in preterm birth, low birth weight (LBW), or small for gestational age [10]. In 2014, a study discovered that the IVF babies from mothers with or without OHSS (Ovarian Hyper-stimulation Syndrome) display alterations in heart rate, vascular diastolic functions, and fibromuscular dysplasia compared to the control [11]. Although there is a global recognition of reducing multiple pregnancies following ART [12], a survey of ART in US in 2016 showed that the percentage of multiple-birth infants was higher among infants conceived with ART (31.5%) than among all infants born in the total birth population (3.4%) [13]. Multiple pregnancies have higher risks for adverse birth outcomes, such as preterm birth (< 32 weeks of gestation) and very low birth weight (< 1500 g) [14,15]. Besides the perinatal consequences, ART-treated offspring may be more vulnerable to cardiovascular, neurological and metabolic diseases in adulthood [16]. Both ICSI children and IVF children have been found significantly increased systemic blood pressure (BP) as compared to the control group [17,18]. Furthermore, under stressful conditions of high-altitude exposure, right ventricular dysfunction was detected in the adolescents conceived by ART [19,20]. Besides this, embryo/gametes cryopreservation in the early stage of life may have an important impact on the developmental process of the whole lifetime. Frozen blastocyst transfer has been associated with a high incidence of pre-eclampsia recently [21]. Pre-eclampsia is associated with abnormal placentation that could lead to subtle macro-vascular and cardiac

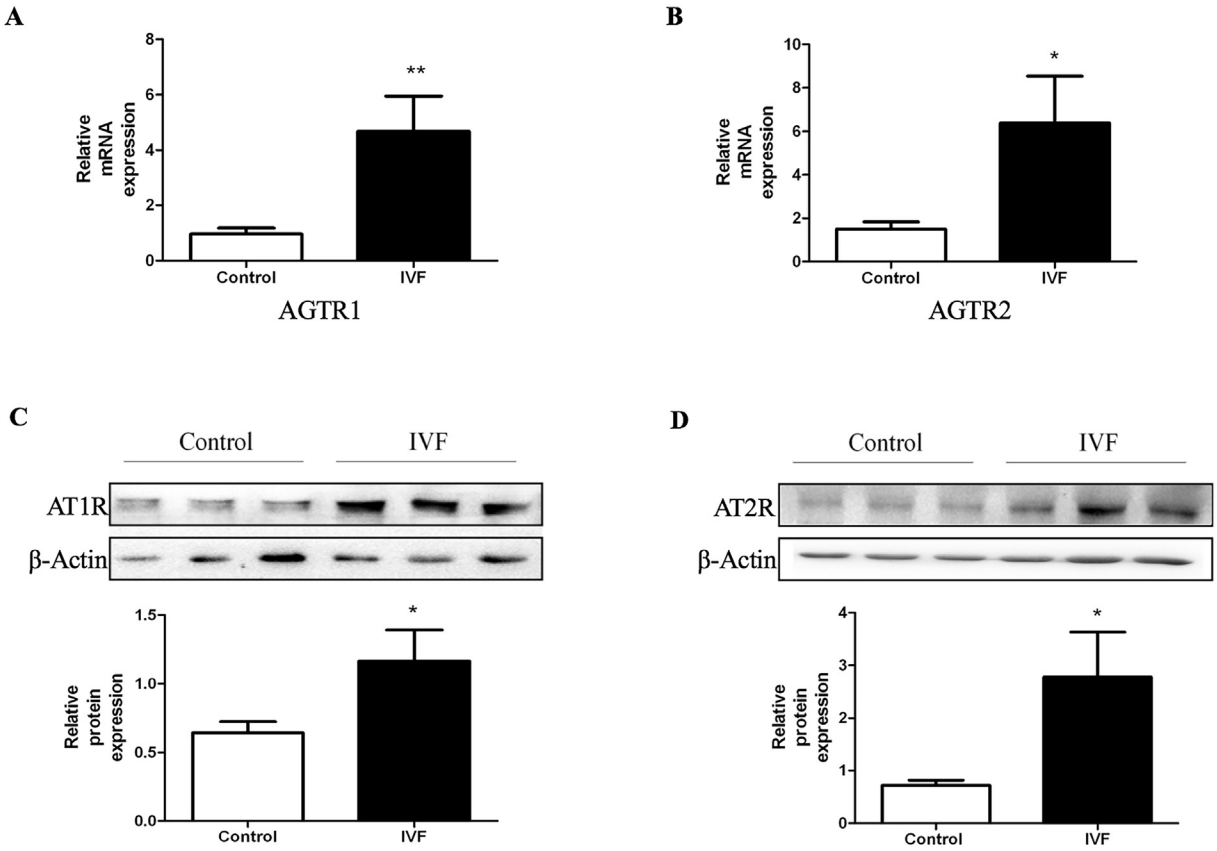


Fig. 3. (A) The mRNA expression of AGTR1 was significantly increased in the IVF-ET group. (**, $p = 0.0069$, two-tailed unpaired Student's *t*-test, $n = 20$. Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.) (B) The mRNA expression of AGTR2 was increased in the IVF-ET group. (*, $p = 0.216$, two-tailed unpaired Student's *t*-test, n = 20. Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.) (C) The protein level of AT1R was significantly increased in the IVF-ET. (*, $p = 0.0386$, two-tailed unpaired Student's *t*-test, n = 10, Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.) (D) The protein level of AT2R was significantly increased in the IVF-ET. (*, $p = 0.0112$, two-tailed unpaired Student's *t*-test, n = 10, Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.)

changes in fetal structures and functions. Exposure to maternal hypertensive disorders in pregnancy is associated with higher blood pressure and body mass index (BMI) after birth [22]. However, how ART may affect fetal vascular functions in human is unknown, which is the focus of the present study.

Renin-angiotensin system (RAS) plays an important role in maintaining body fluid balance and regulating blood pressure. The abnormal activation of RAS under pathological conditions will increase risks of hypertension [23]. For example, maternal low protein diet during pregnancy could increase the risk of hypertension in the offspring via altered methylation of angiotensin receptor [24]. In the present study, fetal blood vessels (umbilical cord veins) were used to explore whether the IVF-ET procedure could affect the RAS system in fetal vascular systems, and the potential epigenetic mechanism was further discussed.

2. Materials and methods

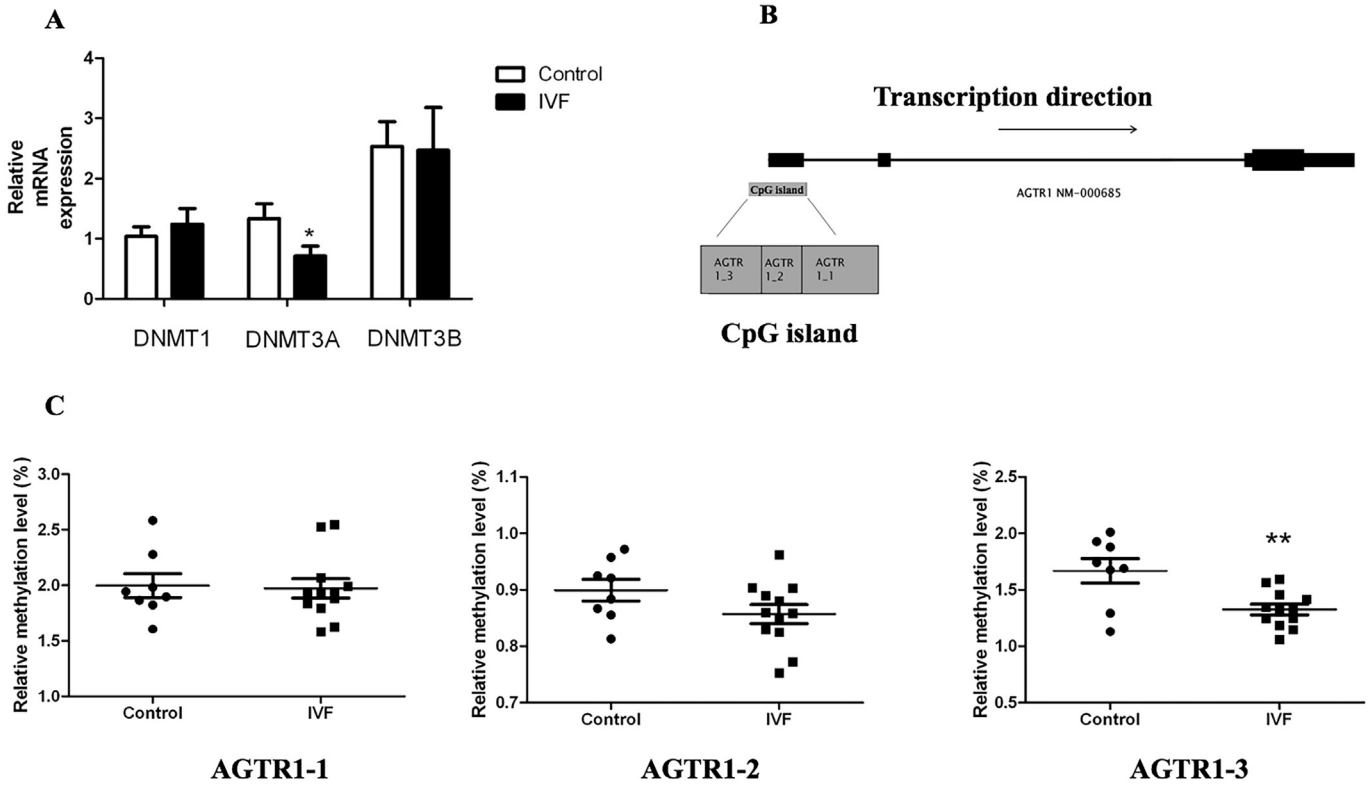
2.1. Human samples

Umbilical cords of ordinary pregnancy (N = 43) or pregnancy of IVF-ET (N = 33) were obtained from the local hospitals, Suzhou, China. Informed consent regarding all experiments was obtained from participants, in accordance with the Declaration of Helsinki (2013) of the World Medical Association. The study was approved by Ethics Committee of the First Hospital of Soochow University (ref. no. 2011-118). Ordinary pregnancies were defined as those with blood pressures < 140/90 mmHg with no significant complications. Pregnancy of IVF-ET is the process of taking eggs, retrieving the sperm sample, fertilization, embryo culture, fresh blastocyst transfer or frozen blastocyst transfer to the uterus (Supplementary Table 1). Umbilical cord veins were immediately collected after delivery. Umbilical cord veins were carefully isolated for vessel functional studies, and umbilical cord tissues were stored at −80 °C until analyses.

2.2. Vascular studies

Isolated human umbilical veins were immediately mounted in an organ bath, containing modified Krebs solution (in mM: 115.0 NaCl, 25.0 $NaHCO_3$, 4.6 KCl, 2.5 $CaCl_2$, 1.2 Na_2HPO_4, 1.2 $MgCl_2$, and 10.0 D-glucose; pH 7.4), bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Vascular tension was measured with JZ101 isometric force transducer (Xinhangxingye Technology, Beijing, China). After 60 min of equilibration, each vessel ring was stretched for the optimal resting tension determined by the tension developed in response to 60.0 mM KCl. Vascular responses to addition of angiotensin II (AII) was monitored and recorded.

Rings of umbilical cord veins were prepared by giving an initial tension and adjusted to that tension for 1 h using KCl (0.12 mol/L). Vasoconstrictions were induced by cumulative additions of AII, and were normalized to the KCl-elicited contractions. MS40001 tension detector and Digital Signal Conversion Instrument was used to measure vasoconstrictions in HUV. Induced vasoconstrictions were obtained following cumulative addition of AII (10^{-11}–10^{-5} mol/L). After pretreating umbilical rings for 20 min with the angiotensin receptor subtype 1 (AT1R) inhibitor losartan (10 μmol/L) or subtype 2 (AT2R) inhibitor PD123,319 (10 μmol/L), AII-mediated contractions were tested.



Target	P-value	Con(%)	IVF(%)	Methylation
AGTR1-1	0.85658	1.9976±0.3011	1.9726±0.298	(-)
AGTR1-2	0.120543	0.8995±0.0541	0.8575±0.0579	(-)
AGTR1-3	0.0048247	1.6696±0.3079	1.3302±0.1655	↓

Fig. 4. (A) DNMT3A expression levels were decreased in the IVF-ET pregnancy, whereas there was no difference in DNMT1 and DNMT3B. (*, $p = 0.04$, two-tailed unpaired Student's *t*-test, n = 15, Control: ordinary pregnancy; IVF: in vitro fertilization-embryo transfer pregnancy.) (B) Three regions from CpG islands of AGTR1 were sequenced. (C) One of the three sequenced region (AGTR1-3) was found decreased in methylation level, the rest two regions (AGTR1-1, AGTR1-2) were no significant difference compared with ordinary pregnancy. The total methylation level of AGTR1 was decreased in the IVF group compared with the Control group. (**, $p = 0.0048$, two-tailed unpaired Student's *t*-test, Control: ordinary pregnancy, n = 8; IVF: in vitro fertilization-embryo transfer pregnancy, n = 12).

All drugs used from Sigma (St. Louis, USA) were freshly prepared.

2.3. Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from umbilical cord veins using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Purified total RNA (500 ng) was then reversely transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. All primers used were listed in Supplementary Table 2. q-PCR was performed with gene-specific primers, cDNA and SYBR Premix Ex Taq (TaKaRa) using a Bio-Rad icycler iQ. Each assay was repeated 3 times and the relative levels of mRNA were normalized to the control actin using the $2^{-\Delta\Delta CT}$ method.

2.4. Western blotting

Human umbilical vein samples were homogenized in 500 μL RIPA buffer added with PMSF, on ice for 30 min. After centrifugation at 13,800*g* for 30 min at 4 °C, clean extracts were obtained and subjected to Western blot analysis. Protein (50 μg) were loaded to 10% SDS-PAGE gels and transferred to PVDF membrane. The membranes were incubated with the antibodies AT1R (Santa Cruz, 1:500); AT2R (Abcam, 1:1000); β-actin (Beyotime, 1:1000) overnight at 4 °C. After washing with Tris-buffered saline with Tween (TBS-T), the membranes were incubated with secondary antibodies (goat anti-mouse for AT1R, goat anti rabbit for AT2R, 1:1000) for an hour. The protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA). Results were quantified using a UVP Bio-imaging system EC3 apparatus (UVP, Upland, CA, USA). AT1R and AT2R protein abundance was assessed by normalized to β-actin.

2.5. Methylation analysis

Genomic DNA from umbilical vein was extracted using phenol: chloroform: isoamyl alcohol (25:24:1, Solarbio) and treated with sodium bisulphite with EZ DNA MethylationTM-GOLD Kit (ZymoResearch) according to manufacturer's protocols. Unmethylated cytosine residues were converted to thymines, whereas methyl-cytosines remain unmodified. For targeting CpG regions, PCR amplification was completed. After PCR amplification and library construction,

products were sequenced on the Illumina MiSeq platform according to the manufacturer's protocols. Methylation levels of 69 sites in AGTR1 (gene encoding AT1R) were measured. Each tested CpG site was named according to its relative distance (in bp) to transcriptional start site (TSS). The percentage of methylation level of each CpG site was calculated as the ratio of methylated cytosines/total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the gene.

2.6. Statistical analysis

Data are presented as the mean ± SEM, and analyzed using GraphPad Prism, version 5.0 (GraphPad Software, San Diego CA). Data were analyzed with a two-tailed unpaired Student's *t*-test or two-way ANOVA followed by Bonferroni post hoc tests. $P < 0.05$, was considered statistically significant. The results were expressed as mean ± SEM.

3. Results

3.1. Impact of IVF-ET on birth body weight

The average gestational weeks of in vitro fertilization-embryo transfer pregnancy (38 weeks ± 3.768 days) is shorter than those in ordinary pregnancy (39 weeks ± 1.013 days) (Fig. S1A). Body weight of singleton new-born babies was measured immediately after birth. 3198 ± 17 g in IVF group and 3297 ± 56 g in the control, indicating that in vitro fertilization-embryo transfer could reduce fetal body weight ($p < 0.001$) (Fig. S1B).

3.2. AII mediated vasoconstrictions in umbilical cord veins

AII caused dose-dependent vasoconstrictions in human umbilical vein. Following KCl-induced (60.0 mmol/L) maximum constriction, the maximal response to AII at a dose of 10^{-5} in IVF-ET group was significantly greater than that in the control. These results demonstrated that the umbilical cord vein of IVF-ET to AII was significantly sensitive than those from ordinary pregnancy ($P < 0.001$). (See Fig. 1.)

3.3. Roles of AII receptor antagonists in umbilical vasoconstrictions

To identify angiotensin receptor subtypes mediating the contraction, the specific receptor antagonists, losartan (AT1R antagonist) and PD123,319 (AT2R antagonist), were incubated. In the IVF-ET group, incubation of losartan increased AII-induced contraction significantly more than that in the control; while PD123,319, the AT2R inhibitor, exerted no different effects between IVF-ET and ordinary pregnancy. The result indicated that the AT1R was mainly involved in the enhanced constriction in the umbilical vein of the IVF-ET pregnancy ($p < 0.001$) (See Fig. 2).

3.4. Expression of AII receptors in umbilical cord vein

As shown in Fig. 3, compared with umbilical veins of ordinary pregnancy, real-time quantitative PCR showed the mRNA abundance of AGTR1 and AGTR2 (gene encoding AT2R) were significantly up-regulated in the IVF-ET pregnancy. AT1R and AT2R protein was also found to be increased in the umbilical veins of IVF-ET group. The results showed that the both receptors of AII were elevated in the IVF-ET pregnancy.

3.5. Methylation in umbilical cords vein

DNA methylation is an important epigenetic mark regulating gene expression. Therefore, three DNMTs were examined. There was no significant difference between IVF and ordinary group in DNMT1 and DNMT3B mRNA expression. However, mRNA expression of DNMT3A was decreased in IVF-ET group compared with the control. Three regions in CpG islands of AGTR1 were selected for further methylation analysis. Methylation level within AGTR1-3 region was found decreased, while the rest two regions (AGTR1-1 and AGTR1-2) were no significant difference when compared with ordinary pregnancy. A decrease in the average methylation levels of AGTR1 was found in the IVF group ($P < 0.01$), suggesting that AGTR1 was hypo-methylation in the umbilical cord vein of the IVF-ET group (See Fig. 4).

4. Discussion

More and more evidences suggested that adult chronic diseases such as cardiovascular, metabolic or neurological diseases have been developed in fetal origins, which is referred as fetal origin of adult disorders (FOAD) by Dr. David Barker [25]. Children born following the use of ART technology such as IVF-ET may be undergoing non-nature handling on eggs, sperm, and embryos in the laboratory. These performance always raised concerns over whether those kinds of operations could affect the babies in long-term. Recently, studies have suggested that the ART can show detrimental effects on the offspring's cardiovascular development [26]. A study performed on sheep demonstrated that the maternal undernutrition before and during the first week of twin pregnancies resulted in an increase in fetal arterial pressure [27]. The umbilical cord is physiologically part of the fetus and in human and it normally contains two arteries and one vein. The umbilical arteries supply deoxygenated blood from the fetus to the placenta. In umbilical cord vein, one of the branches bypasses the liver and flows into the inferior vena cava, which carries blood towards the heart. This study used umbilical cord veins to determine outcomes in fetal blood vessels in IVF-ET pregnancy, exploring possible evidence if fetal vascular functions might be influenced by ART.

The present study was the first to show that the RAS system and its functions in fetal umbilical vessels were disrupted following IVF-ET pregnancy, as the maximal vascular response to AII was significantly greater than that in the ordinary pregnancy. Angiotensin II is a peptide hormone that causes vasoconstrictions and plays a critical role in regulating blood pressure [28]. Our result showed that the umbilical cord veins under conditions of IVF-ET procedure tended more sensitive in vasoconstrictions than that in the control. Thus, the data obtained could be viewed as the new evidence that the IVF-ET procedure may affect fetal cardiovascular systems. Angiotensin II is a major signaling molecule of the RAS, acting on various subtypes of AII receptors. In order to determine which subtype pathway involved in the umbilical vasoconstriction differences between the IVF-ET and control groups, the receptor subtypes were tested and measured. Compared with ordinary pregnancy, the expression of both AT1R and AT2R were significantly elevated in umbilical veins of IVF-ET group. AT1R is a major subtype mediating AII-induced vasoconstrictions. The physiological and biological effects of AII, including vasoconstrictions, increase of BP, cardiac contractility, oxidative stress and endothelial dysfunction, are mainly depended on AT1R [29]. On the other hand, effects of AT2R are relatively unclear, even though there were suggested opposite effects of AT1R [30]. If so, the up-regulation of AT2R found in our study may be a compensated adjustment to the increasing of AT1R in the fetal vessels.

The formation of 5-methylcytosine (5mC) is catalyzed by DNA methyltransferase (DNMTs), which occurs primarily at cytosine-guanine dinucleoelated and usually results in transcriptional repression. DNMT1 takes charge for the maintenance of methylated DNA, while DNMT3A and DNMT3B are the de novo DNA methyltransferase that work on non-methylated DNA. The present study found that DNMT3A was significantly decreased in the umbilical vein in IVF-ET group, demonstrating that IVF-ET treatment might have impact on the methylation regulation with the genome DNA in the ART-treated babies. Furthermore, in IVF-ET group, AGTR1 was found hypo-methylated, which could consequently cause the up-regulation of this gene. These

results are consistent with the higher sensitivity to AII found in fetal umbilical vessels with the ART treatment, suggesting interestingly that ART could lead to changes in fetal vessel functions through the epigenetic regulation.

In mammals, the fertilization takes place in mothers' oviduct, where providing a unique environment for gametes to encounter and develop in the first stage of the embryo. This is a period of major epigenetic reprogramming, and is extremely vulnerable to environmental insults or changes, while IVF treatments are considered significant changes of the environment, including in vitro culture, nutrition, light, temperature, oxygen tension, embryo-maternal signaling, and so on [31–35]. The whole stability of this development process could be affected by the environment factors and the absence of the maternal protection [36,37]. Previous research found that exposure to plasticware such as leakage of xenoestrogens, phthalates and from plastic polymers during the IVF implantation period could alter the uterine morphology, estrogen and progesterone receptors, and result in a lower implantation percentage [38]. Those plasticware exposures have been shown in vivo and in vitro studies to induce epigenetic alterations, including cytosine methylation and/or histone acetylation [39]. Making a general view of the whole IVF-ET operation, in vitro culture (IVC) is probably the most crucial and relevant factor in the alterations of epigenetic reprogramming and development of animal embryos produced. A large number of studies confirm that negative influence of the culture medium such as the effects of proteins, quality of the water and serum in epigenetic preimplantation reprogramming and its adverse impact on early embryo development [40–42]. For decades, it has been described that animals produced via IVF show abnormally large birth size and organ size, named as large offspring syndrome (LOS). All the LOS features such as large size at birth, gross abnormalities in various organs, are similar to those found in the Beckwith–Wiedemann syndrome (BWS). BWS is an imprinting disorder, usually representing over-growth at birth, characterized by an increased risk of childhood cancer and certain congenital features. Some children with BWS, specific parts of the body on one side or the other may grow abnormally large, leading to an asymmetric or uneven appearance. In fact, LOS was found to be associated with the absence of methylation at the KvDMR1, the control region regulating imprinting genes responsible for BWS [43].

We believe that ART may have benefit effects for pregnancy as several reports demonstrated before, which is not a barrier for study of possible side effects of ART [5, 6]The present study found that the hypo-methylation of AII type 1 receptors was associated with an increased expression of AT1R mRNA and protein. In rat models, the increase in expression of that receptor protein accompanied by an increase in AII could eventually induce hypertension [24,44]. BMI is a key parameter that may influence blood pressure and hormones such as angiotensin II levels during pregnancy [45]. We realized that was a weakness of the current study, not including measurement of BMI. This important factor should be considered in future studies. Our study showed, in the IVF-ET group, umbilical cord veins were more irritable to AII stimulation, and the related receptors were increased too. Those changes, in long-term, may add risks in hypertension and other cardiovascular diseases in the offspring. Despite more studies are required for further evaluation possible effects of IVF on fetal as well as offspring vascular systems, the data gained in the present study presented interesting and critical information for clinical practice of IVF and babies' long term health under those conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116792.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Methylation-reprogrammed AGTR1 results in increased vasoconstriction by angiotensin II in human umbilical cord vessel following in vitro fertilization”.

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