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The increased expression of glucose transporters in human full-term placentas from assisted reproductive technology without changes of mTOR signaling

Jie Dong[1], Liang Wen[1], Xiangyu Guo, Xifeng Xiao, Feng Jiang, Bo Li, Ni Jin, Jingjing Wang, Xin Wang, Shuqiang Chen**, Xiaohong Wang*

Department of Obstetrics and Gynaecology, Tangdu Hospital, Air Force Medical University, Xi'an, 710032, Shaanxi, China

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ABSTRACT

Objective: In the mouse model, manipulations of assisted reproductive technology (ART) can lead to enlarged placentas and influence the expression of glucose transporters (GLUTs) in placentas during mid-to late-gestation. Expression of imprinted genes which plays a vital role in placental growth and function, is also vulnerable to be affected by ART. However, it is uncertain whether those abnormal changes presented in ART mouse placentas also occur in human ART placentas.

Methods: We compared the expression of GLUT family genes (*SLC2A1- SLC2A13*), mTOR activity, the expression of four imprinted genes (*H19*, *IGF2*, *CDKN1C* and *PHLDA2*), and *KCNQ1OT1* methylation in human placentas conceived naturally or by ART.

Results: Our data showed that the placental weight and birthweight were similar between NC (n = 20) and ART group (n = 20). We found that up-regulated mRNA expression of GLUTs and elevated GLUT1 protein level occurred in human ART placentas with unchanged mTOR activity. And we found that mRNA and protein expression of PHLDA2 were significantly increased in ART placentas compared with placentas from natural pregnancies. Additionally, we revealed that ART placentas had increased expression of *KCNQ1OT1* which negatively controls *PHLDA2* expression.

Conclusion: This study reveals that the increased expression of GLUTs occurs in human ART placentas with normal mTOR activity. The down-regulated expression of imprinted gene PHLDA2 may account for the up-regulation of GLUTs. Those adaptive changes in ART placentas may explain why most of ART offspring have normal birth weight at born.

1. Introduction

Epidemiological data and experimental studies have indicated that critical disruption to fetal development *in utero* results in cardiovascular or metabolic disease in adulthood [1]. This phenomenon is termed fetal programming, because fetal organs are said to undergo "programming" during development that can lead to permanent physiological and metabolic changes postnatally [1]. The placenta plays a vital role in this process by mediating the maternal-fetal resource allocation. Furthermore, the fetal environment is the result of multiple placental functions, including the directional transport of nutrients and oxygen, the secretion of cytokines, hormones, and growth factors, and the adaptive responses to environmental cues [2]. When the fetus is exposed to a stressful environment, caused by problems like malnutrition, hypoxia, and infection, the placenta adapts by changing its blood flow and the surface area exchange and transporter activity to ensure that the placental supply of nutrients and oxygen can meet the fetal demand [3,4]. However, according to the hypothesis of Developmental Origins of Health and Disease (DOHaD), these placental adaptations may cause alterations to the fetal composition and result in life-long programming, contributing to the occurrence of hypertension, diabetes, and cardiovascular diseases later in life [5].

During fertilization and preimplantation, the embryos undergo gradual demethylation, which is called "epigenetic reprogramming"

* Corresponding author. Department of Obstetrics and Gynaecology, Tangdu Hospital, Air Force Medical University, Xi'an, 710032, Shaanxi, China.
** Corresponding author. Department of Obstetrics and Gynaecology, Tangdu Hospital, Air Force Medical University, Xi'an, 710032, Shaanxi, China.
E-mail addresses: chenshuqiang2012@163.com (S. Chen), wangxh-99919@163.com (X. Wang).
[1] These authors contributed equally to this article.

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[6]. Manipulations of assisted reproductive techniques (ART) are mainly performed during this critical period. The conventional ART process, mainly including *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), exposes gametes or preimplantation embryos to non-physiological conditions, which can lead to perturbations in epigenetic patterns (such as DNA methylation and genomic imprinting) [7,8]. So far, more than 6 million babies have been born via ART worldwide [9]. Although the majority of individuals conceived by ART are healthy, many studies have indicated that ART children have higher risks of cardiovascular dysfunction and changes in glucose metabolism, which suggests that fetal programming may be occurring during ART pregnancies [10–12]. Considering the critical role of the placenta in fetal growth and programming [3,13], studies have focused on the changes of ART-derived placentas in humans and have shown abnormal epigenetic modifications resulting from ART [14–16]. Additionally, Haavaldsen et al. [17] found that human ART placentas present increased weight compared to those derived from natural conception in late gestation. However, not many studies report on whether the functions of human placentas are changed by ART.

Using mouse models, multiple studies have found that IVF placentas display profound increases in size and weight during mid-to late-gestation, whereas IVF fetuses exhibit restricted growth in early-to mid-pregnancy while catching up to the growth of naturally conceived fetuses by late gestation [18]. In addition, the expression and DNA methylation of imprinted genes (*H19*, *Phlda2*, *Peg3* and *Kcnq1ot1*) in IVF placentas significantly differ from those of control placentas, and the expression levels of glucose transporters (GLUTs) are also different [19–21]. These results suggest that mouse placentas generated by ART may undergo excessive growth and functionally adaptive alterations to meet the needs of ART fetuses during intrauterine development. Whether these adaptive placental changes in ART mice translate to human ART pregnancies still remain unknown.

Therefore, we hypothesized that adaptive changes may occur in the human ART placenta and that imprinted genes may be involved in these placental changes. In this study, the placental expression of GLUT family genes and mTOR signaling were measured to determine whether the nutrition environment was affected in ART placentas. Moreover, we examined the expression of several vital imprinted genes in the placenta. Finally, considering that our previous study have shown that *KCNQ1OT1* expression is down-regulated in mouse ART placentas [19], we compared the mRNA expression and methylation level of long non-coding RNA *KCNQ1OT1,* which negatively regulates imprinted gene *PHLDA2* expression in placentas [22]. Collectively, our study revealed that ART placentas showed increased GLUTs expression and decreased PHLDA2 expression, which indicates that ART placentas may suffer from adaptive responses.

2. Materials and methods

2.1. Sample collection

This study was approved by the Tangdu Hospital Ethics Committee. Placentas from ART or natural control (NC) were collected with the informed consent of patients who delivered from July 2014 to June 2015. ART samples were collected from pregnancies conceived by IVF or ICSI, and NC samples were harvested from spontaneous pregnancies. All samples were obtained from singleton pregnancies at term without pregnancy complications, including gestational hypertension, preeclampsia, gestational diabetes, and birth defects. Placental tissues were obtained within half an hour after delivery of the placenta by a gynaecologist, nurse, or midwife. The biopsies (~5 mm^3) were obtained from the fetal side near the umbilical cord insertion site, washed extensively in cold phosphate-buffered saline to remove blood, immediately frozen in liquid nitrogen, and stored at −80 °C for later use.

2.2. RNA extraction, cDNA synthesis and quantitative PCR (RT-qPCR)

Total RNA was extracted using the Trizol reagent (Invitrogen, MA, USA) according to the manufacturer's instructions and treated with DNaseI to remove DNA contamination. The purity of RNA was determined by a Nanodrop 2000 spectrophotometer (Thermo, MA, USA). A total of 1 μg of total RNA was converted to cDNA using a commercially available cDNA Reverse Transcription (RT) Kit (Takara, Shiga, Japan). RT-qPCR was performed using a CFX connect real-time PCR instrument (Bio-Rad, CA, USA). Each cDNA sample was diluted 5-fold. The reaction mixture for RT-qPCR consisted of 1.5 μl of diluted cDNA, 0.75 μl of each primer (0.1 μM), 10 μl of SYBR premix Ex Taq™ II (Takara, Shiga, Japan) and 7 μl of nuclease-free water. All samples were run in duplicate. RT-qPCR amplification was performed under the following conditions: 95 °C for 3 min, 45 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 2 min. The expression of each target gene was normalized to that of the reference gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)*. The primer sequences were obtained from Primer Bank or designed with the Primer Blast tool and listed in Supplemental Table 1. We accepted that the Ct value of each gene in each well after PCR amplification was below 35. The fold change in expression of each gene was analyzed using the ΔΔCt method [23].

2.3. DNA methylation mass array

Genomic DNA from the placentas was extracted using a Blood and Tissue DNA Kit (Qiagen, Hilden, Germany). DNA quality and quantity were determined by agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (Thermo), respectively. A total of 1 mg of DNA was treated using an EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. PCR primers of *KCNQ1OT1* imprinting control region (ICR) were designed using EpiDesigner software (Supplemental Table 2). The methylation levels of *KCNQ1OT1* ICR were analyzed by MassArray, which combines base-specific enzymatic cleavage with MALDI-TOF mass spectrometry. This method for methylation detection allows the evaluation of multiple CpG sites on a single amplicon and is quite accurate, sensitive and reproducible. The amplified DNA fragments were examined on a MassArray platform (Sequenom, CA, USA) at the Beijing Genomics Institute of Shenzhen. T7 promoters were used in the process of locus-specific PCR amplification to generate *in vitro* transcripts. These transcripts were then treated for enzymatic RNA base pair cleavage. The end products had different masses and sizes depending on the sequence changes resulting from bisulphite treatment. The fragment mass was handled by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). The methylation level at each CpG site or aggregates of multiple CpG sites was determined with EpiTYPER software. The average methylation levels were calculated using the weighted average.

2.4. Western Blot

Placental protein was extracted using RIPA lysis buffer (Millipore, MA, USA). The concentration was measured using a Bradford Assay Kit (Thermo). We used Coomassie Brilliant Blue Staining (SimpleBlueTM, Invitrogen) and ACTIN expression to determine the quality of protein samples and adjust the loading volume of protein specimens. Proteins were separated using 4–12% or 10–20% SDS Tris-glycine gels (Invitrogen) and then transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies. Primary antibodies were purchased from Abcam including GLUT1 (ab115730, 1:1000), GLUT3 (ab191071, 1:1000) and ACTIN (1:5000, Abcam, #119716), Antibodies from Cell Signaling Technology (CST, MA, USA) included phospho-mTOR Ser2448 (p-mTOR, #5536, 1:1000), total mTOR (t-mTOR, #2983, 1:1000), phospho-P70S6K Thr389 (p-P70S6K, #9205, 1:1000), total P70S6K (t-P70S6K, #2708, 1:1000), phospho-rpS6 Ser235/6 (p-

rpS6, #4858, 1:1000), total rpS6 (t-rpS6, #2217, 1:1000), phospho-4EBP1 Thr37/46 (p-4EBP1, #2855, 1:1000) and total 4EBP1 (t-4EBP1, #9644, 1:1000). PHLDA2 antibody was obtained from Bioss (1:400, #bs-6884R, Beijing, China). ACTIN was used as the normalizing control. The secondary antibodies were HRP-conjugated goat anti-rabbit antibodies (1:5000, #7074, CST). Bands were visualized using an enhanced chemiluminescence kit (Millipore). Image Lab 6.0 software was used to analyze the bands.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. The quantitative variables are described as the means ± standard derivation (SD). The comparisons of continuous variables with normal distribution between two groups were analyzed with Student's *t*-test while continuous variables with skewed distribution were compared by Mann-Whitney test. Binary variables were compared with the χ^2 test. A *P*-value < 0.05 was considered significant.

3. Results

3.1. Clinical characteristics of the samples

A total of 20 IVF/ICSI and 20 NC placental samples were harvested in this study. All of the samples were collected from singleton pregnancies at term without gestational complications. As shown in Table 1, the baseline characteristics including maternal age, gestational age, maternal body mass index (BMI), parity, delivery mode and neonatal sex showed no significant differences between the ART group and the NC group. We compared the birth weight, placental weight, and placental efficiency (measured by the ratio of birthweight to placental weight) between the ART group and the NC group. We found that there were no significant differences between the two groups (Table 1).

Table 1
Maternal and Neonatal characteristics.

	NC (N = 20)	ART (N = 20)	*P* Value
Maternal age, years (SD)	30.9 (3.8)	32.6 (4.9)	0.228
Gestational age, days (SD)	277.1 (5.9)	277.4 (5.7)	0.871
Maternal BMI, kg/m^2 (SD)	21.24 (0.9)	21.32 (1.2)	0.812
Parity			0.407
1	15	18	
> 1	5	2	
Mode of delivery			0.056
Vaginal delivery	13	6	
Cesarean section	7	14	
Newborn sex			1.000
Boy	10	11	
Girl	10	9	
Infertility cause			
Female	NA	16	NA
Male	NA	4	NA
ART technique			
IVF	NA	16	NA
ICSI	NA	4	NA
Transferred embryo, n			
1	NA	1	NA
2	NA	18	NA
3	NA	1	NA
Birth weight (SD), g	3501.5 (385.1)	3464.0 (411.6)	0.768
Placental weight (SD), g	564.0 (89.8)	561.5 (83.4)	0.928
Placental efficiency (SD)	6.3 (1.0)	6.2 (0.8)	0.787

Data were described as mean (standard deviation) or numbers. NA: not applicable. Continuous variants and binary variants were analyzed using Student's *t*-test and χ^2 test, respectively.

3.2. Expression levels of GLUTs in the placentas

The relative mRNA levels of ten out of fourteen GLUT genes were measured, as the four remaining GLUTs (*SLC2A2*, *SLC2A4*, *SLC2A7*, and *SLC2A14*) could not be stably detected using the RT-qPCR. Compared with the NC placentas (n = 20), the mRNA expression level of four GLUTs (*SLC2A1*, *SLC2A3*, *SLC2A8*, and *SLC2A11*) were significantly upregulated in the ART placentas (n = 20) (Fig. 1a, b, e, h). The other GLUTs (*SLC2A5, SLC2A6, SLC2A9, SLC2A10*, *SLC2A12* and *SLC2A13*) did not show significant differences at the transcriptional level between the NC and ART placentas (Fig. 1c, d, f, g, i, j). Additionally, because GLUT1 (SLC2A1) and GLUT3 (SLC2A3) are the two main glucose transporters in human placenta [24], we compared the protein expression of GLUT1 and GLUT3 between NC (n = 15) and ART group (n = 15). The data showed that GLUT1 protein level was significantly higher in ART placentas (Fig. 2a) than in NC placentas while the GLUT3 protein level had an increased trend in ART placentas without statistical significance (Fig. 2b). The increased expression of GLUTs in ART placentas indicated that changes in glucose transport in ART placentas may occur.

3.3. mTOR activity in the placentas

The mTOR signaling pathway plays a central role in sensing placental glucose metabolism and regulates the nutritional balance to support fetal growth [25,26]. Because we found the increased expression of GLUTs in ART placentas, we hypothesized that mTOR activity in the ART placentas could be over-activated. We used the ratio of the phosphorylated protein level to the total protein level to evaluate mTOR activity. The phosphorylation activity of key molecules in mTOR signaling pathway were analyzed, including mTOR, P70S6K, rpS6 and 4EBP1. Different from our expectation, we found that there were no significant differences in phosphorylation level of mTOR, P70S6K, rpS6 and 4EBP1 between ART and NC placentas (Fig. 3a–d).

3.4. Expression levels of imprinted genes in the placentas

We examined the expression levels of four imprinted genes, including one maternally imprinted gene (*IGF2*) and three paternally imprinted genes (*H19, CDKN1C* and *PHLDA2*). The expression levels of *H19*, *IGF2* and *CDKN1C* (Fig. 4a–c) were similar between the ART (n = 20) and NC groups (n = 20), but the expression level of *PHLDA2* was significantly decreased in the ART placentas (Fig. 4d). Furthermore, we evaluated the protein level of PHLDA2 using Western Blot and found that the protein level of PHLDA2 was also significantly reduced in the ART placentas (n = 15) compared with the NC placentas (n = 15) (Fig. 4e).

3.5. Expression and methylation level of KCNQ1OT1 in the placentas

Because the expression of *PHLDA2* in the placenta has been shown to be regulated by the long non-coding RNA *KCNQ1OT1* [22], we sought to determine whether the expression level of *KCNQ1OT1* in ART placentas was changed. We found that the mRNA level of *KCNQ1OT1* was significantly higher in ART placentas (n = 17) than in NC placentas (n = 18) (Fig. 5a). *KCNQ1OT1* expression is controlled by the methylation degree of its imprinting control region (ICR), thus we sought to analyze the methylation level of *KCNQ1OT1* ICR. In this test, the methylation levels of 60 CpG units of *KCNQ1OT1* ICR within the target sequences were measured. However, we found that the mean methylation level of the CpG sites in *KCNQ1OT1* ICR only had a decreased trend in ART placentas (n = 20) compared to NC group (n = 19) (Fig. 5b). With regard to the methylation levels of single CpG sites in *KCNQ1OT1* ICR, the whole single CpG sites also did not show significant differences between the ART placentas and NC placentas (Fig. 5c). According to these results, we inferred that the decreased

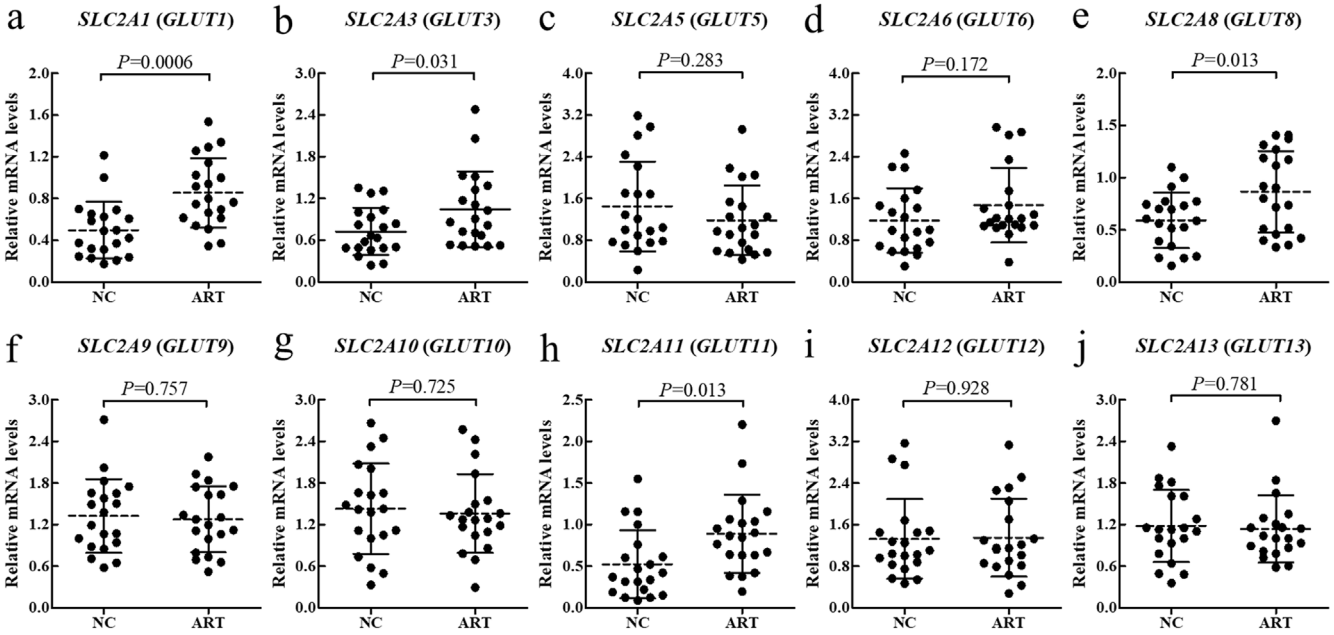

Fig. 1. The relative mRNA expression of GLUT family genes (*SLC2A1*, *SLC2A3*, *SLC2A5*, *SLC2A6*, *SLC2A8-SLC2A13*) in placental tissues from NC (n = 20) and ART (n = 20) pregnancies. Error bars represent standard deviation.

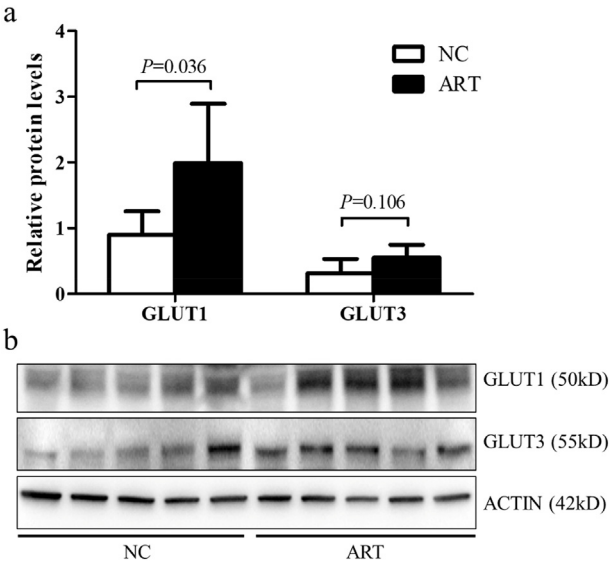

Fig. 2. The relative protein expression of GLUT1 and GLUT3 in placental tissues from NC and ART pregnancies. a: The protein level of GLUT1 and GLUT3. b: The blots of GLUT1, GLUT3 and ACTIN protein. Each blot was derived from mixture of three different samples. Error bars represent standard deviation.

expression level of PHLDA2 in the ART placentas may be related to the increased expression of *KCNQ1OT1* mRNA.

4. Discussion

Glucose, the principal energy substrate for the placenta and the fetus, is essential for fetal development and growth. The glucose transfer from the placenta to the fetus mainly depends on the function of GLUTs in the placenta [24,27]. In this study, we found that human ART placentas had increased expression of GLUTs, indicating a possible change in the placental glucose transfer mechanism. Also, the mTOR signaling pathway plays an important role in sensing placental glucose metabolism and regulates the nutritional balance to support fetal growth [25,26]. In the placenta, mTOR responds to a variety of growth stimulating factors, including amino acids, glucose, oxygen, and folate, to coordinate nutrient transport, trophoblast mitochondrial respiration and protein synthesis, thereby affecting fetal growth [28]. In addition, the mTOR pathway is associated with the expression of GLUTs. Studies have shown that GLUT3 expression was markedly decreased in JEG-3 trophoblast cells in response to mTORC1 silencing [29], and the increased glucose uptake and GLUT1 levels were blocked by inhibition of mTOR with rapamycin [30]. Because we found increased expression of GLUTs in ART placentas, we initially hypothesized that mTOR activity in the ART placentas could be over-activated. However, our study found that the mTOR signaling in ART placentas remained unchanged, indicating that changes in the glucose transport may be occurring in human ART placentas with no changes to the mTOR activity.

To further understand what other factors may be involved in the nutritional environment changes of ART placentas, we examined the expression of several imprinted genes, including *PHLDA2*, which has been previously shown to be negatively correlate with the growth of the placenta and the fetus [31,32]. Imprinted genes play vital roles in placental development and function [7]. Past studies have shown that placentas generated by ART exhibit imprinting disturbances and changes in the overall expression of imprinted genes [7,33]. Our study confirmed these past findings by showing the decreased expression of *PHLDA2* in ART placentas. Our results further supplied the reasoning that the decreased expression of *PHLDA2* may be associated with the increased expression of *KCNQ1OT1* and that there are no changes to the methylation of *KCNQ1OT1* ICR during this process. Ultimately, the down-regulated expression of the imprinted gene *PHLDA2* may account for the upregulation of GLUTs.

ART is considered to be a stress factor for embryos due to the non-physiological manipulations of the process, which may disrupt the pattern of fetal development *in utero* and result in high risks for pregnancy complications [34–36]. Past studies have suggested that the abnormal changes in epigenetic modifications caused by ART procedures may be responsible for adverse pregnancy outcomes [7,14]. Moreover, studies have demonstrated that DNA methylation errors may persist in the placenta but not in fetal tissues at mid-gestational stage in mice [37,38]. However, no study has yet to fully determine what specific changes occur in human ART placentas. A population study by Haavaldsen et al. [17] investigated birthweights and placental weights

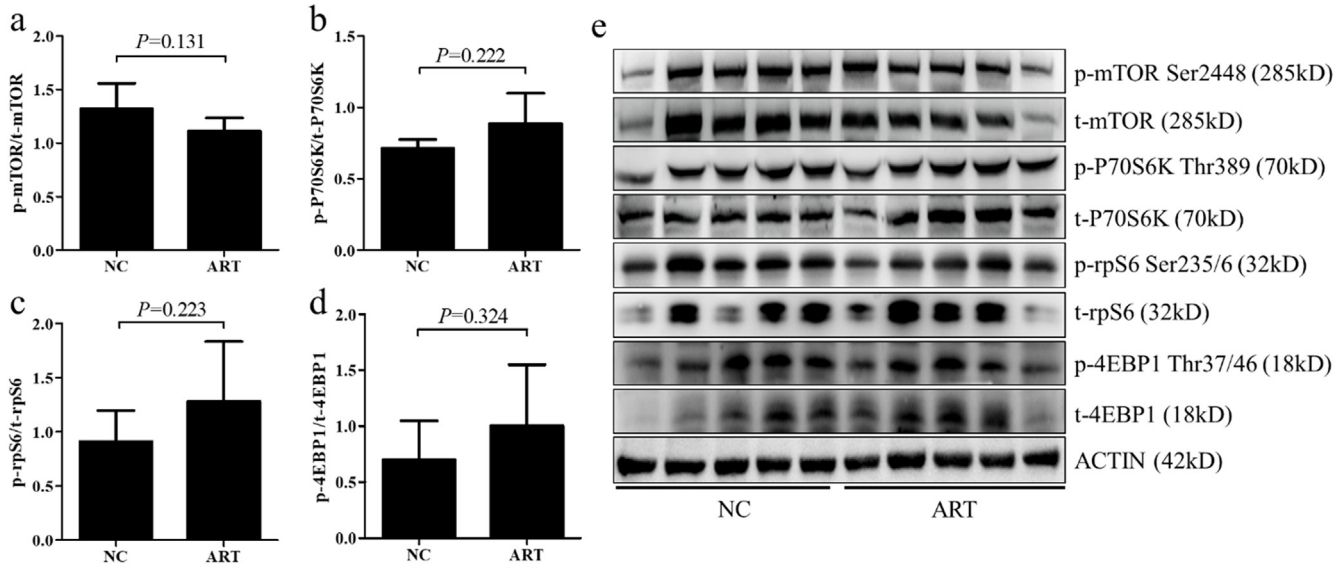

Fig. 3. The phosphorylation activity of key molecules in mTOR pathway in placentas from NC and ART pregnancies. a–d: The relative phosphorylation level of mTOR, P70S6K, rpS6 and 4EBP1, respectively. e: The blots of protein including p-mTOR, t-mTOR, p-P70S6K, t-P70S6K, p-rpS6, t-rpS6, p-4EBP1, t-4EBP1 and ACTIN. Each blot was derived from mixture of three different samples. Error bars represent standard deviation.

from 536,567 pregnancies and found a very small but significant increase in the placental weight of ART placentas compared with those from natural pregnancies. However, Yanaihara et al. [39] reported that there was no difference in the size of the placenta between women with natural pregnancy (n = 1453) and with IVF (n = 157). Our study supported the results of Yanaihara et al. [39] as we did not find any significant differences in placental weight between the ART and NC group. More prospective studies with larger cases should be performed to confirm this point and settle the conflicting results about the differences in placental weight of ART and NC patients.

Although there was no change in the placental size between ART and NC groups, we noted altered expression levels of GLUTs in ART placentas. Our study showed that the mRNA expression levels of four GLUTs (*GLUT1*, *GLUT3*, *GLUT8* and *GLUT11*) and GLUT1 protein expression were significantly higher in ART placentas. These results indicate that ART placentas may undergo adaptive alterations to glucose transport without bringing changes to the placental weight in ART pregnancies, which is strikingly different from the observations made by past IVF mouse models. In mouse models, IVF leads to larger size of placentas with reduced expression of GLUTs and decreased weight of newborns [19–21]. These varying results between humans and mice suggest that these two species may have different regulatory

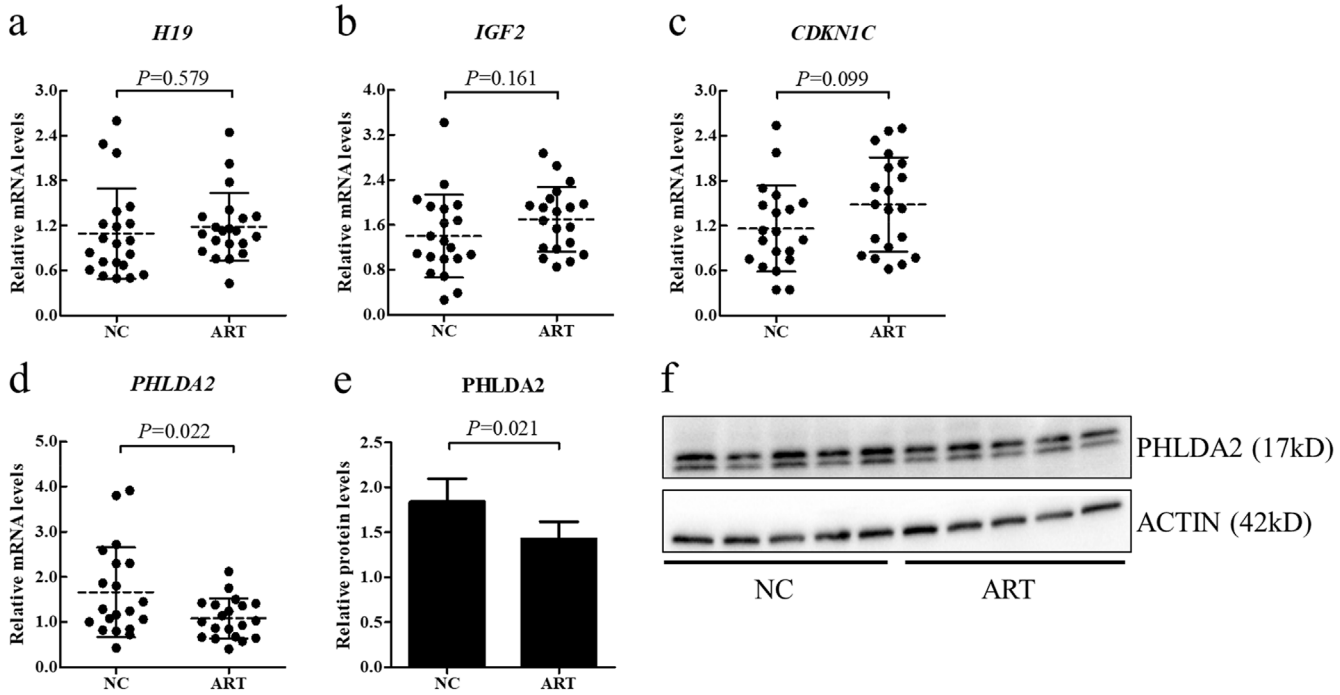

Fig. 4. The expression of imprinted genes in placentas from NC and ART pregnancies. a–d: The relative mRNA level of *H19*, *IGF2*, *CDKN1C* and *PHLDA2*, respectively. e: The relative protein level of PHLDA2. f: The protein blots of PHLDA2 and ACTIN. Each blot was derived from mixture of three different samples. Error bars represent standard deviation.

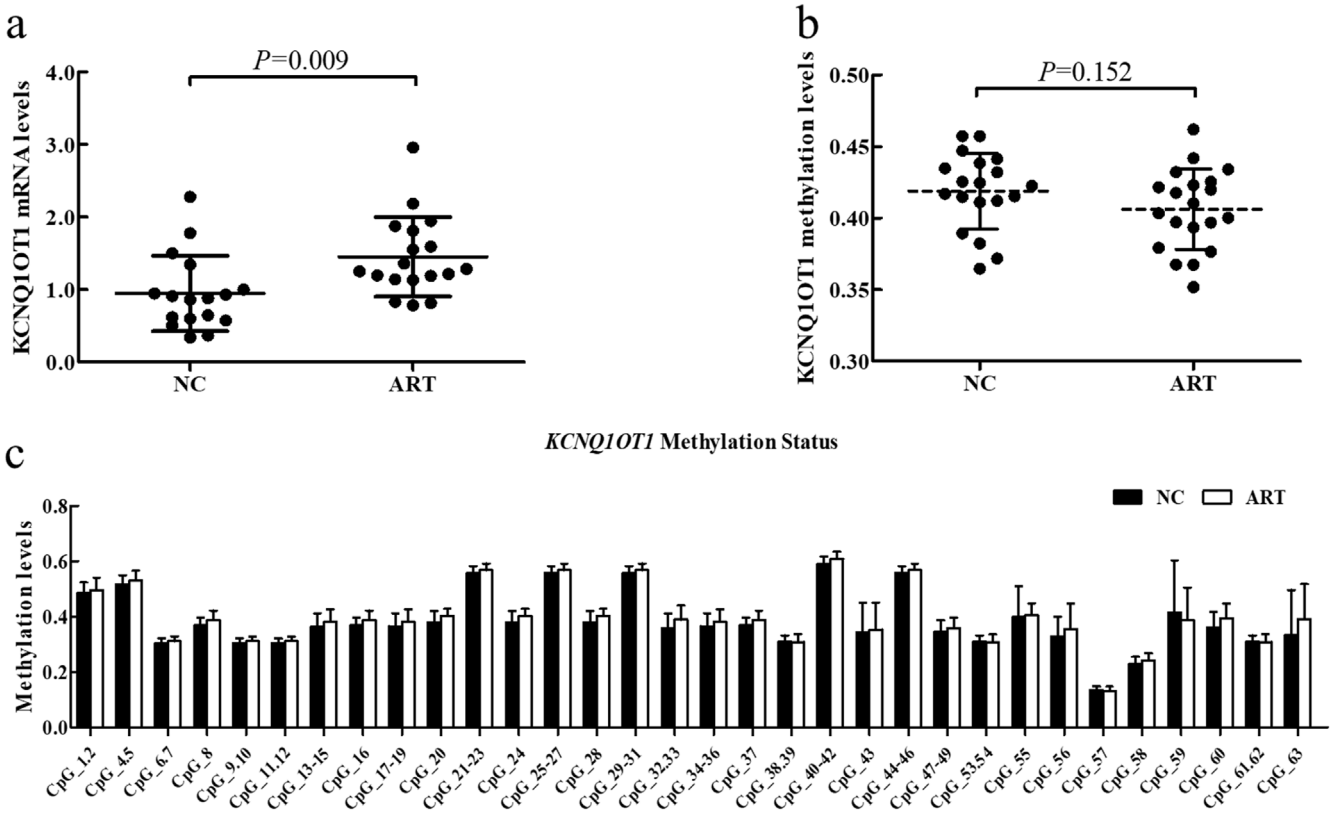

Fig. 5. The level of *KCNQ1OT1* mRNA expression and methylation. a: The relative expression of *KCNQ1OT1* mRNA. b: The mean global methylation level of *KCNQ1OT1* ICR. c: The methylation level of individual CpG site in *KCNQ1OT1* ICR. Error bars represent standard deviation.

mechanisms that modulate the adaptive responses in ART placentas. To ensure that the fetuses conceived by ART are receiving sufficient nutritional supply, the mouse may compensate for placental capabilities via placental enlargement, while humans may compensate for placental capabilities by upregulating the expression of nutrient transporters. Further research studying the species differences between humans and mice ART models must be done to understand this speculation.

The causes of changes in the expression levels of placental GLUTs still remain unclear, but epigenetic changes may explain part of this process. Studies have shown that genomic imprinting plays an important role in placental growth and function [40] and that ART manipulations can cause disruption of imprinting modifications in mouse placentas [37,38]. Studies in humans have also revealed that ART is correlated with imprinting aberrations and abnormal expression of imprinted genes in the placenta [15,41,42]. Studies have reported that PHLDA2 is inversely linked to intrauterine growth in humans [31,32], regulates placental glycogen storage, and affects expression of Glut in mice [43]. As a result, it is reasonable to assume that PHLDA2 may be negatively correlated with GLUT expression in human ART placentas. In support of this idea, our study found that the mRNA and protein level of the imprinted gene *PHLDA2* was significantly reduced in ART placentas, similar to the finding observed by Feng et al. [42]. However, a study conducted by Nelissen et al. [44] have shown that the mRNA expression of *PHLDA2* is increased in human IVF/ICSI placentas although the protein level of PHLDA2 was not measured in this study. In addition, most of the placental tissues in the study by Nelissen et al. [44] were obtained from ICSI pregnancies, while the samples in our study were mainly from IVF subjects. At present, there is no definite evidence showing that ICSI can lead to a higher risk of adverse pregnancy outcomes or placental insufficiency when compared to IVF. Therefore, the reason for the different results in *PHLDA2* expression in ART placentas may be due to different geographic environments and life-styles leading to different genomic backgrounds in the participants. Further studies are essential for investigating the role and mechanism of PHLDA2 in the regulation of GLUT expression.

As an imprinted gene, *PHLDA2* expression is negatively associated with the regulation of *KCNQ1OT1* mRNA [22]. We found that the expression of *KCNQ1OT1* mRNA was significantly higher in ART placentas than in NC placentas, which indicates that the upregulation of *KCNQ1OT1* mRNA may contribute to the low expression of *PHLDA2* in ART placentas. Furthermore, we analyzed the methylation level of *KCNQ1OT1* ICR, which regulates the expression of *KCNQ1OT1* mRNA, and found that the methylation level of *KCNQ1OT1* ICR showed a decreasing trend in ART placentas without statistical significance. Several studies have shown that hypomethylation at *KCNQ1OT1* ICR can be observed in ART placentas [45–47]. Our study results on the methylation changes of *KCNQ1OT1* in ART placentas may differ from other studies due to our small sample size or because we tested different DNA fragments. Therefore, the decreased expression of *PHLDA2* in ART placentas may be the result of *KCNQ1OT1* expression being upregulated due to its lower methylation levels.

In summary, our study shows that the changes in GLUTs and PHLDA2 expression occur in ART placentas with normal mTOR signaling. These changes are likely associated with imprinting disorders in ART placentas. Our study provides further mechanistic explanation for the idea that functional alterations in the glucose transport of human ART placentas may occur to allow the ART fetus to obtain the same amount of glucose supply as normal pregnancies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.08.087.

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