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Altered expression of mTOR and autophagy in term normal human placentas

QIU XIA ZHANG, QUAN NA, WEIWEI SONG

Department of Obstetrics and Gynecology, Shengjing Hospital, China Medical University, Shenyang, China

Abstract

Mammalian target of rapamycin (mTOR) and autophagy have been implied in trophoblast cells proliferation and invasion. This study investigated whether mTOR and autophagy were involved in placental development and fetal programming. A total of 90 term normal placentas (37–42 gestational weeks) were collected from women who underwent elective cesarean section, with large for gestational age (LGA), fetal growth restriction (FGR), and appropriate for gestational age (AGA) infants (n=30, respectively). Capillary volume density within peripheral villi significantly decreased in the FGR placentas compared with the AGA group (p<0.01). Autophagic vacuoles were more prominent in the FGR placentas than in the AGA pregnancies (p<0.001). LC3B-II and Beclin1 in placentas of FGR were increased by 99% and 83%, respectively, while p62 was descended by 39% (p<0.001), compared with that in the AGA group. Whereas, there was no significant differences in these autophagy-related proteins between the LGA and AGA pregnancies. Compared to the AGA pregnancies, placental mTOR was reduced by 30% in the FGR group and raised by 26% in the LGA group (p<0.05). Pearson's correlation analysis showed a significantly inverse correlation between mTOR level and LC3B-II expression (r=-0.456, p<0.01), as well as between mTOR and Beclin1 expression in term human placentas (r=-0.468, p<0.01). These results demonstrate that altered expression of mTOR and autophagy may be associated with the development of placentas and pathophysiology of FGR, and that there may be a reciprocal regulation between mTOR and autophagy in human placentas.

Keywords: autophagy, mTOR, placenta, fetal growth restriction.

Introduction

Abnormal birth weight is associated with a variety of diseases, including type 2 diabetes mellitus, hypertension, hyperlipidemia and cardiovascular disease [1, 2]. Fetal growth restriction (FGR), defined as fetal birth weight at or below the tenth percentile for sex and gestational age, has been identified to be associated with a high incidence of perinatal morbidity and mortality [3]. Additionally, according to the recent research, newborns of FGR are at a higher risk of developing acute neonatal diseases, childhood neurodevelopmental dysfunction, and adult metabolic syndromes [3]. Thus, it is of great significance to study the pathophysiology of altered fetal growth.

Placenta is of critical importance for fetal programming, as it exchanges a wide array of nutrients, endocrine signals, cytokines and growth factors with the mother and the fetus [4, 5]. Notably, abnormal development and dysfunction of placentas, due to disorders in trophoblasts cell proliferation, differentiation and invasion, can cause suboptimal fetal outcomes [4]. Although the etiology for FGR is not fully understood, placental insufficiency is the most widely recognized predisposing factor for FGR [6], its molecular pathology remains, however, elusive.

Mammalian target of rapamycin (mTOR), a catalytic component of two complexes, mTORC1 and mTORC2, is a conserved protein kinase that regulates many fundamental biological processes, such as cell growth and proliferation, by coordinating an adequate response to changes in energy uptake (amino acids, glucose), growth signals (hormones, growth factors) and environmental stress [7]. It has been implicated in an increasing number of pathological conditions, including cancer, metabolic disorders and neurodegeneration [8, 9]. In addition, mTOR is essential for growth and proliferation of early mouse embryos and embryonic stem cells [10, 11]. Recent studies have implicated its role in trophoblast proliferation and invasion [12, 13]. Therefore, we suspect that the expression of placental mTOR may be related to development of placenta and fetal growth.

Autophagy is a vacuolar process of cytoplasmic degradation by lysosome, which produces amino acids, free fatty acids and other substances for energy and protein synthesis [14]. Proper autophagy is more widely viewed as a basic cell survival mechanism to combat various environment factors, such as nutrient and growth factor deprivation, and endoplasmic reticulum stress, while over-activated autophagy is considered to accelerate the cascade of cellular death [15, 16]. These functions of autophagy favor the adaptation of cells and promote cellular survival during aging, infections, and neurodegenerative processes [17].

Concerning the correlation between autophagy and placenta, a few studies focused on the expression of autophagy-related proteins in human placentas [18–22]. Placentas obtained from cesarean section exhibited a higher level of autophagy than vaginal delivery [19]. Abnormal autophagy is proposed to account for poor placentation in preeclampsia [18, 20]. Furthermore, *in vitro* studies have recently reported that autophagy has a great impact

on trophoblast functions [20, 21]. However, few studies have addressed the role of autophagy in placental development and fetal birth weight.

mTOR has been well established as a critical regulator of autophagy in various model systems [23, 24]. However, the relation between mTOR and autophagy in human placentas has not yet been elucidated. In the present study, we examined the expression of mTOR and autophagy in term human placentas associated with altered fetal growth, thereby investigated the associations among mTOR, autophagy and fetal birth weight.

Materials and Methods

Ethic statement

The research was ethically approved by the research ethics committee of Shengjing Hospital affiliated with China Medical University [No. 2010PS (55)]. All placental samples were collected after the subjects provided written informed consent.

Placenta sample collection and preparation

Recent studies show that delivery mode and pregnancy complication have an impact on autophagy in human placentas [18, 19]. Thus, we collected 90 term normal placentas (37–42 gestational weeks) from women who had elective cesarean section. According to altered birth weight, they were divided into three groups, including large for gestational age (LGA), fetal growth restriction (FGR), and appropriate for gestational age (AGA) (n=30, respectively). The diagnosis of FGR and LGA infants were based on the standard definition as birth weight in the 10^{th} and 90^{th} percentile for fetal sex and gestational age, respectively. A detailed history, physical examination, laboratory tests, and systematic ultrasonic measurements of biparietal diameter (BPD), occipitofrontal diameter (OFD), and femur length (FL) were also considered when establishing the clinical diagnosis. Table 1 summarizes the characteristics of the 90 subjects.

Table 1 – ***Characteristics of the study population***

	LGA (n=30)	AGA (n=30)	FGR (n=30)
Maternal age [years]	28.42± 4.46	29.53± 6.21	29.24± 5.04
Pre-pregnancy BMI [kg/m^2]	23.82± 3.53	21.91± 4.38	22.43± 3.72
Gestational age [weeks]	38.89± 0.73	39.01± 0.54	39.32± 0.91
Birth weight [g]	4179± 172***	3239± 339	2274± 163***
Placental weight [g]	966± 99***	748± 102	467± 79***
Primiparous (n)	21	18	16

BMI: Body mass index; LGA: Large for gestational age; AGA: Appropriate for gestational age; FGR: Fetal growth restriction. Data are presented as mean ± SD. ***p<0.001 *versus* AGA.

Right after the placenta was delivered and weighed, we obtained tissue samples from the neighborhood of the umbilical cord and midway between the chorionic and basal plates. The placental samples were quickly washed in saline solution and then stored separately: for Hematoxylin and Eosin (HE) staining, immunohistochemistry and immunofluorescence, tissues were placed into 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin wax; for electron microscopy, samples were fixed in 2.5% glutaraldehyde; for Western blotting, samples were kept at -80^{0}C for protein extraction.

Electron microscopy

Placental samples were cut into 2-mm pieces within 10 minutes of delivery, placed into fixative (2.5% glutaraldehyde) for 12 hours, and then washed in 0.1 M PBS (pH 7.4) twice for 15 minutes. Afterwards, the tissues were postfixed in 1% OsO_4, dehydrated in ethanol plus propylene oxide, and embedded in epoxy resin. Ultrathin sections of 50 to 60 nm were then stained with uranyl acetate for 30 minutes and lead citrate for 10 minutes. A Jeol JEM 1010 (Tokyo, Japan) electron microscope was used to capture images. Autophagic vacuoles were defined as double membrane vesicles containing cell components. For each case, the area of autophagic vacuoles was calculated by two observers on 60 000× printed micrographs in three randomly selected areas of placental villi, in trophoblasts, endothelial cells and stromal cells, respectively. Quantification of autophagy was measured as a ratio of autophagic vacuole area to the total cytoplasmic area. The mean ratio from the three areas was considered as the final value for each case [25].

Immunostaining

For histological analyses, paraffin-embedded samples were sectioned (5 μm) and stained with HE according to conventional procedures. In all, 200 random sections of villi per sample were observed under a trinocular microscope (OLYMPUS, CX41) with a 40× objective (approximately 6000 villi for each group). Transparent lattices of test points superimposed on micrographs at random positions were performed to estimate volume density of peripheral villi capillaries [26].

Immunohistochemical studies were conducted on 4-μm thick tissue sections as described previously [27]. Primary antibodies were as follows: anti-LC3B (Novusbio, NB100-2220, 1:500), anti-Beclin1 (Novusbio, NB500-249, 1:400), anti-p62 (Proteintech, 18420-1-AP, 1:500), anti-mTOR (Abcam, ab2732, 1:800). Slides with absence of primary antibodies were considered as negative controls. All the slides were probed with biotin-conjugated goat anti-rabbit secondary antibodies (Dako, 1:200), followed by incubation with avidin-peroxidase (5 μg/mL). Concentrated 3,3'-diaminobenzidine (DAB, Sigma-fast, Sigma) was applied to observe immunostaining. Slides were counterstained with Harris's Hematoxylin and were viewed on a light microscope (OLYMPUS, CX41). All positive cells in the whole villosities were carefully assessed under double-blind conditions. Ten randomly selected fields with 40× objective were taken of each immunomarked slide of placental tissue. The average integrated optical density (IOD) per stained area (μm^2) (IOD/area) for positive staining was calculated by ImageJ software.

Western blotting

Proteins were extracted by using radio-immunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo, 89900) and quantified with the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo, 23227). The 14 000×g supernatant from homogenized samples was diluted with loading buffer, boiled for 5 minutes, and stored at -20^{0}C. Fifty μg of each protein extract was separated by 12% or 6% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis, depending on the molecular weight of the markers studied) and electroblotted on polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The PVDF membranes were incubated in 5% skim milk for one hour at room temperature, and then reacted with primary antibody against human LC3B (Novusbio, NB100-2220, 1:1000), Beclin1 (Novusbio, NB500-249, 1:1000), p62 (Proteintech, 18420-1-AP, 1:1500), mTOR (Abcam, ab2732, 1:1000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, 2118S, 1:1000), at 4^{0}C, overnight. Following incubation with horseradish-peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibody for two hours, at room temperature, bands were visualized on photosensitive film (Amersham Biosciences Ltd., UK), using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences Ltd., UK), and band intensities were quantified using ImageJ software.

Immunofluorescence

Immunofluorescence studies were carried out as described previously [27], using primary antibody against Beclin1 (Novusbio, NB500-249, 1:100). Fluorophore-conjugated secondary antibody (Abbkine, A23220, 1:50) was employed and nuclei were subsequently counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were observed and captured using a confocal laser scanning microscope (Olympus America, Center Valley, PA) using a 40× 1.4 oil immersion lens. Ten randomly selected fields were taken of each immunomarked slide of placental villi. The integrated optical density (IOD)/area for positive cells in the whole villosities was analyzed using the ImageJ software.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and analyzed by using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test, as well as Pearson's correlation test. A p-value <0.05 was considered statistically significant.

Results

Clinical characteristics

A total of 90 samples were enrolled in this study, including placentas with FGR, AGA (control) and LGA fetus (n=30, respectively). The selected clinical data are given in Table 1, in which the differences were statistically validated by the Kruskal–Wallis test followed by the Mann–Whitney *U*-test. Maternal age, pre-pregnancy body mass index (BMI), and gestational age were found not significantly different among the three groups. Birth weight was 30% lower in the FGR group and 29% higher in the LGA group than that in the AGA pregnancies (p<0.001). Similarly, compared to the control group, placental weight was reduced by 38% in the FGR pregnancies (p<0.001) and was increased by 29% in the LGA pregnancies (p<0.001).

Capillary volume density of peripheral villi

Histomorphometrical findings indicated that, compared to the AGA group, the volume density of peripheral villi capillaries in the FGR group was significantly decreased (p<0.01, one-way ANOVA with Bonferroni's *post hoc* test). Whereas, there was no significant difference in the capillary volume density between the LGA and AGA groups (Figure 1, A–D).

Evidence of placental autophagy

Examination of transmission electron microscopy revealed that autophagic vacuoles were located in the trophoblasts (Figure 2A), endothelial cells (Figure 2B) and stromal cells (Figure 2C) of placental villi associated with altered fetal growth. In particular, these autophagic vacuoles containing lysosomal enzymes and semi-digested cell organelles, were more prominent in the FGR placentas, including trophoblasts, endothelial cells and stromal cells, respectively, than the AGA group (p<0.001, one-way ANOVA with Bonferroni's *post hoc* test). Whereas, there were no significant differences in the levels of autophagic vacuoles between the LGA and control groups (Figure 2, A–C).

Expression of placental autophagy

Immunostaining of LC3B was noted in the cytoplasm of trophoblasts, endothelial cells and stromal cells (Figure 3A). Beclin1 was mainly expressed in the cytoplasm and partially in the nucleus of trophoblasts, endothelial cells and stromal cells (Figure 3B). The stainings of LC3B and Beclin1 positive cells in the FGR placentas were more intensive than that in the AGA group (p<0.001; Figure 3, A and B; one-way ANOVA with Bonferroni's *post hoc* test). p62 protein was localized in both the cytoplasm and nucleus of trophoblasts, endothelial cells and stromal cells. Evaluations of p62 protein staining showed an obvious decrease in the FGR placentas compared to the AGA group (p<0.001; Figure 3C). Nevertheless, no significant differences in the LC3B, Beclin1 and p62 stainings were found between the LGA and control placentas (Figure 3, A–C). Immunofluorescence analysis further confirmed the cellular localization of Beclin1 protein and showed that fluorescence intensity of Beclin1 was elevated in the FGR placentas, compared with the AGA group (p<0.001; Figure 4, A and B).

Western blotting analysis (one-way ANOVA with Bonferroni's *post hoc* test) further identified that both LC3B-II and Beclin1 expression were significantly up-regulated by 99% and 83%, respectively, and that p62 exhibited a 39% decline, in the placentas of FGR *versus* AGA (p<0.001; Figure 5, A–D). Protein levels of LC3B-II, Beclin1 and p62 had no significant differences between the LGA and control group (Figure 5, A–D).

Expression of placental mTOR

mTOR was predominantly detected in the cytoplasm and nucleus of trophoblasts, endothelial cells and stromal cells from pregnancies associated with altered fetal growth (Figure 6A). Evaluation of mTOR staining demonstrated that mTOR expression decreased in the FGR placentas (p<0.01) and increased in the LGA placentas (p<0.05), compared with the AGA group (Figure 6B). Immunoblot analysis of mTOR further confirmed the results of the staining evaluation. In comparison to the control group, mTOR protein levels were significantly reduced by 30% in the FGR pregnancies (p<0.01), and raised by 26% in the LGA pregnancies (p<0.05; Figure 6, C and D).

Pearson's correlation analysis showed that mTOR expression was inversely correlated to the expression of LC3B-II (r=-0.456, p<0.01; Figure 7A), and also inversely correlated to Beclin1 expression in term human placentas (r=-0.468, p<0.01; Figure 7B). However, there was no obvious correlation between placental mTOR and p62 expression (r=0.156, p>0.05; Figure 7C).

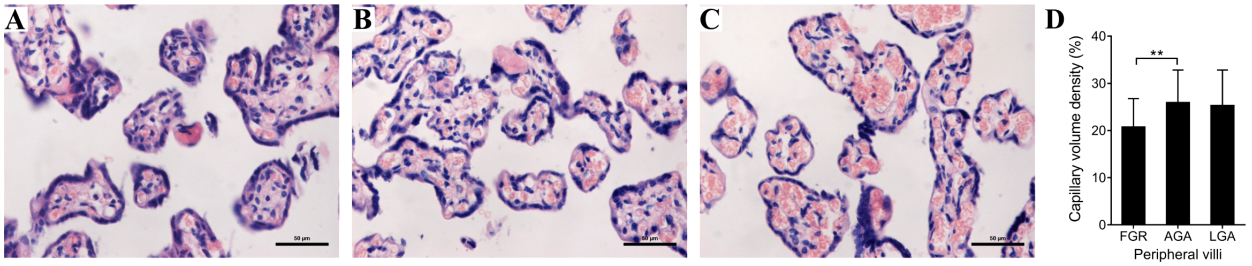

Figure 1 – ***Photomicrograph of histological sections of peripheral villi from pregnancies with FGR (A), AGA (B) and LGA (C) fetus; Magnification ×400; Scale bar: 50 µm. (D) Capillary volume density of peripheral villi was reduced in the FGR placentas versus AGA group, respectively; n=30; **p<0.01, one-way ANOVA with Bonferroni's post hoc test. FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; ANOVA: Analysis of variance.***

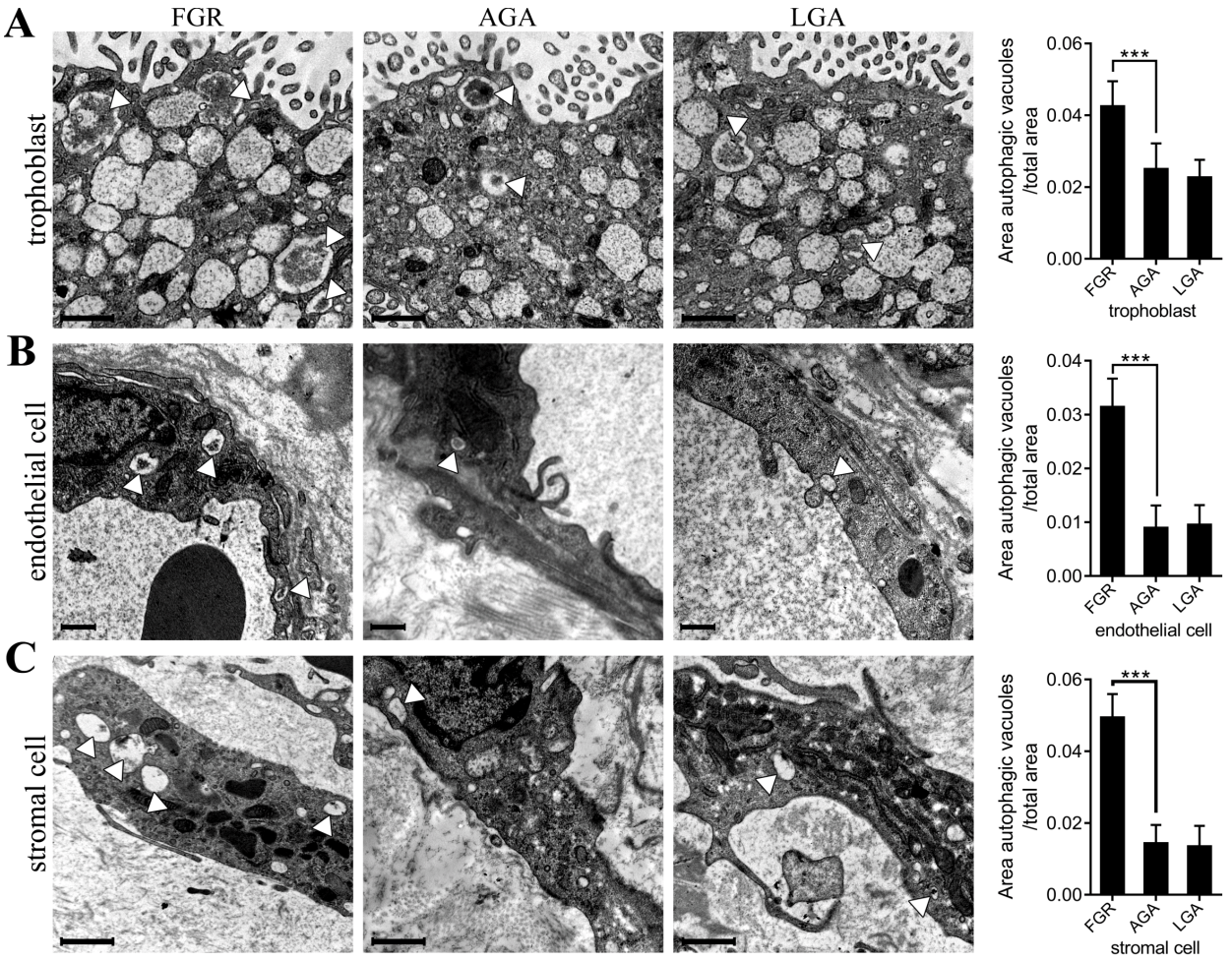

Figure 2 – ***Presence of autophagic vacuoles (arrowheads) in human placentas with FGR, AGA and LGA fetus. Autophagic vacuole content in placental villi, expressed as the ratio of the sum of autophagic vacuole area to the total cytoplasmic area. The concentrations of autophagic vacuoles in trophoblasts (A), endothelial cells (B) and stromal cells (C) were elevated in the FGR placentas versus AGA group, respectively. Scale bars: 1 µm (A and C); 500 nm (B); n=30; ***p<0.001, one-way ANOVA with Bonferroni's post hoc test. FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; ANOVA: Analysis of variance.***

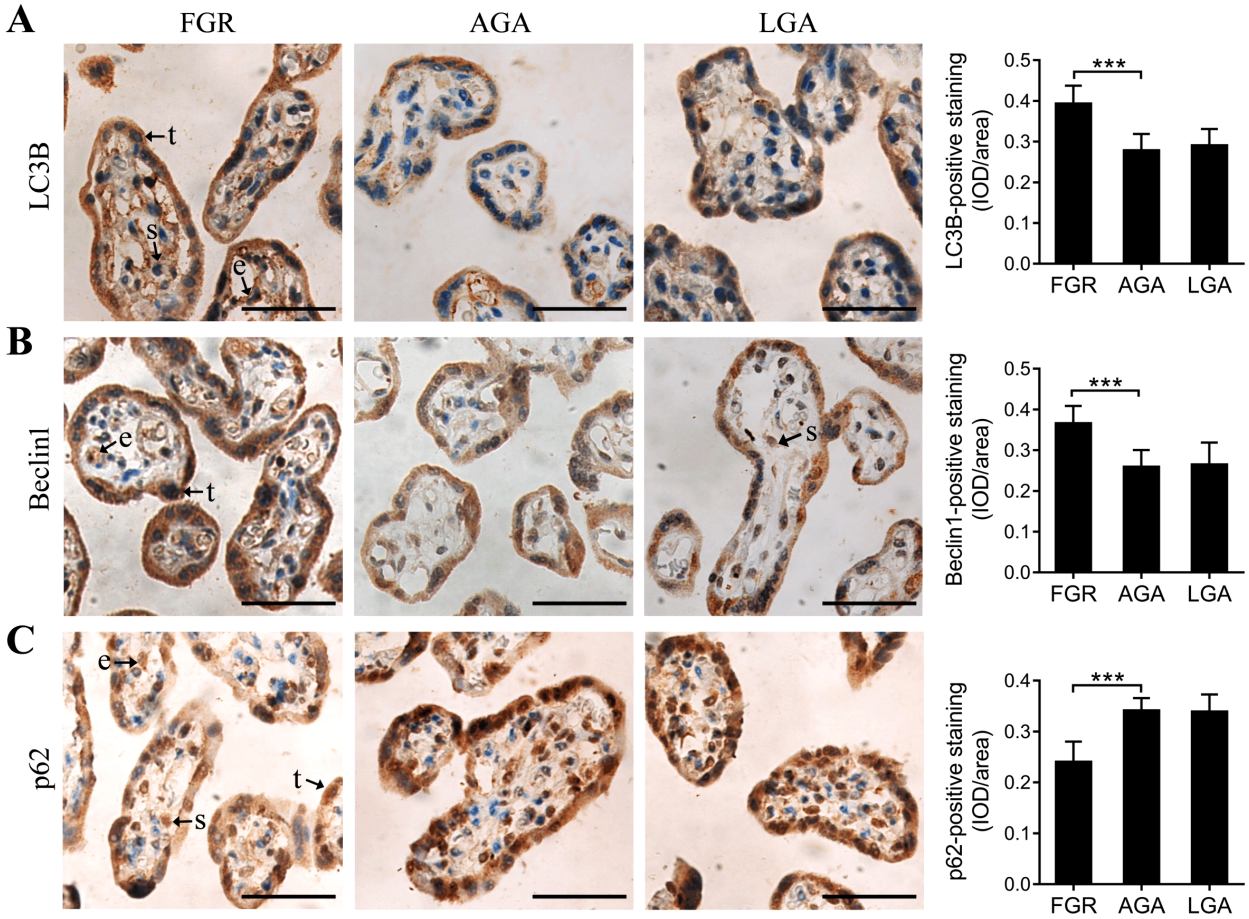

Figure 3 – ***Immunohistochemical expression of LC3B (A), Beclin1 (B) and p62 (C) in human placentas associated with altered fetal growth. The LC3B-positive cells (A) and Beclin1-positive cells (B) significantly increased in the FGR placentas versus control. (C) The p62-positive cells declined in the FGR group, compared to the control. Magnification ×400; Scale bar: 50 µm; n=30; ***p<0.001, one-way ANOVA with Bonferroni's post hoc test. FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; t: Trophoblast; e: Endothelial cell; s: Stromal cell; IOD/area: Integrated optical density per stained area; ANOVA: Analysis of variance.***

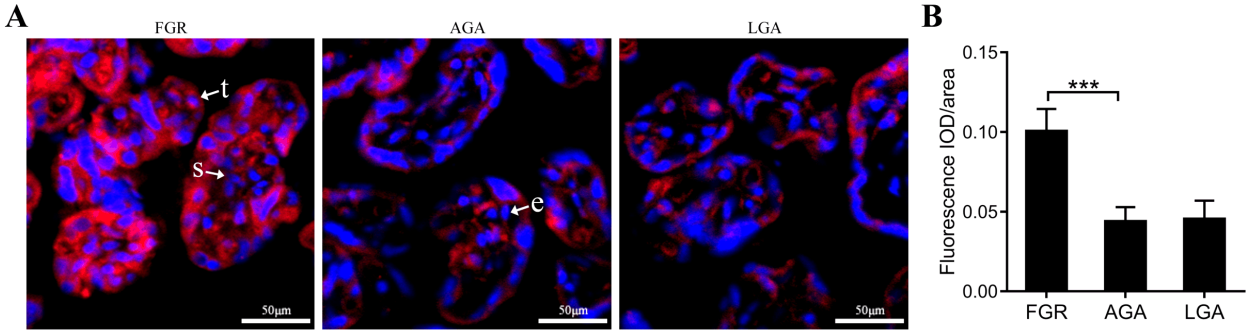

Figure 4 – ***Confocal analysis of Beclin1 expression in human placentas with FGR, AGA and LGA fetus. (A) Beclin1 (red) was mainly localized in the cytoplasm and partially in the nucleus (DAPI, blue) of trophoblasts (t), endothelial cells (e) and stromal cells (s); Magnification ×400; Scale bar: 50 µm. (B) Fluorescence intensity of Beclin1 was enhanced in the FGR placentas versus control. n=30; ***p<0.001, one-way ANOVA with Bonferroni's post hoc test. FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; DAPI: 4',6-Diamidino-2-phenylindole; IOD/area: Integrated optical density per stained area; ANOVA: Analysis of variance.***

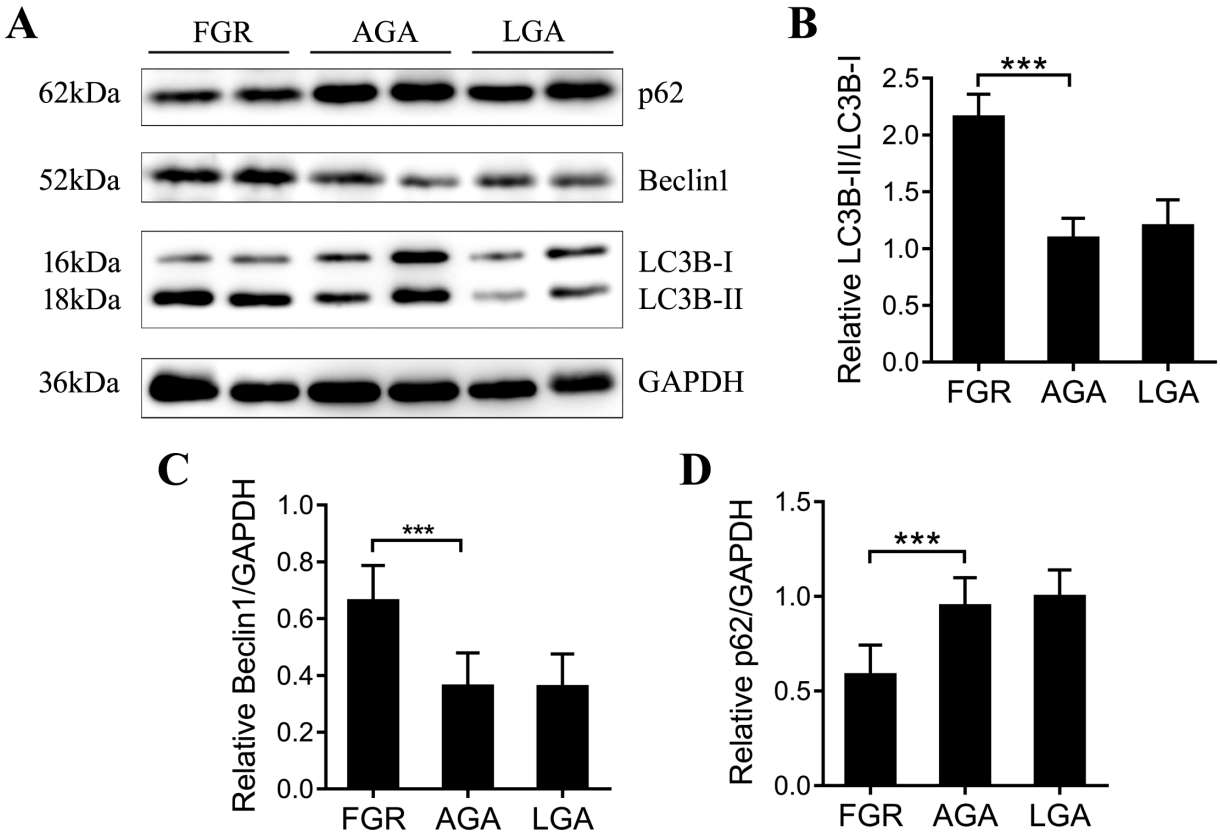

Figure 5 – ***Expression of autophagy-related proteins in the human placentas associated with fetal growth. (A) Western blotting showing distinct bands corresponding to p62, Beclin1, LC3B-I and LC3B-II. GAPDH served as a loading control. (B–D) Quantification of the results in (A). n=30; ***p<0.001, one-way ANOVA with Bonferroni's post hoc test. FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ANOVA: Analysis of variance.***

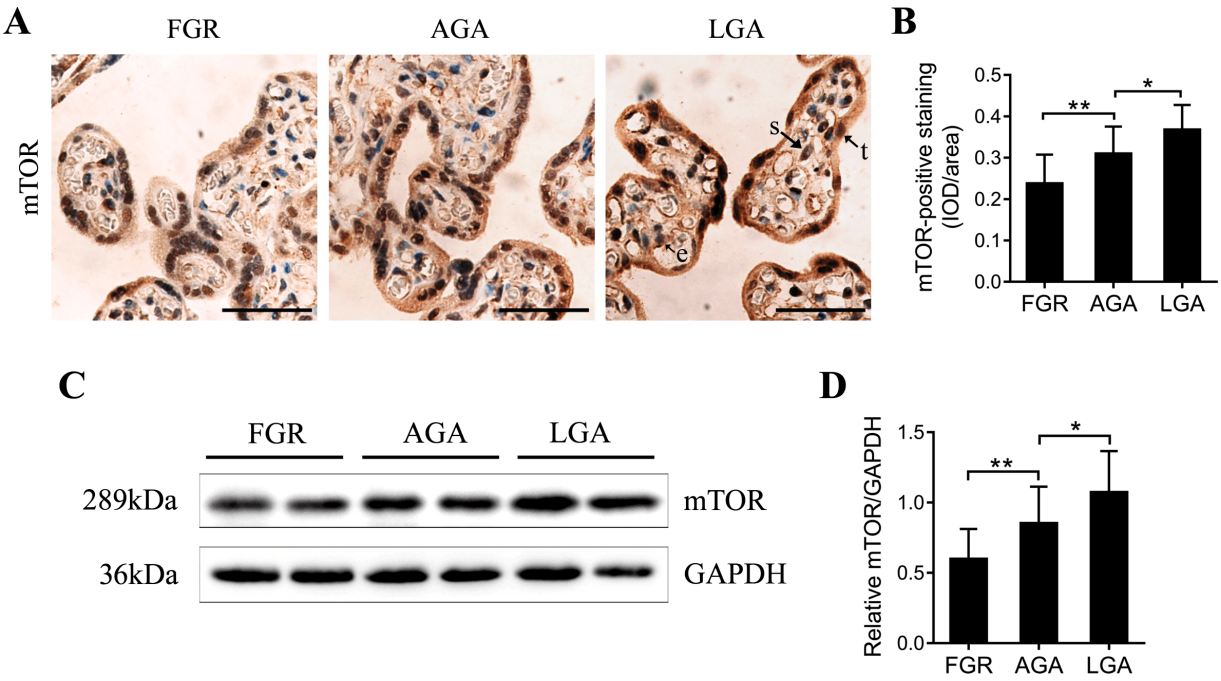

Figure 6 – ***mTOR expression in human placentas with FGR, AGA and LGA fetus. (A) Immunohistochemical staining of mTOR was noted in the cytoplasm and nucleus of trophoblasts (t), endothelial cells (e) and stromal cells (s); Magnification, ×400; Scale bar: 50 μm. (B) mTOR staining in the FGR placentas was more intensive than that in the control group. (C) Western blotting showing distinct bands corresponding to mTOR and GAPDH. (D) Quantification of the results in (C). n=30; *p<0.05, **p<0.01, one-way ANOVA with Bonferroni's post hoc test. mTOR: Mammalian target of rapamycin; FGR: Fetal growth restriction; AGA: Appropriate for gestational age; LGA: Large for gestational age; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IOD/area: Integrated optical density per stained area; ANOVA: Analysis of variance.***

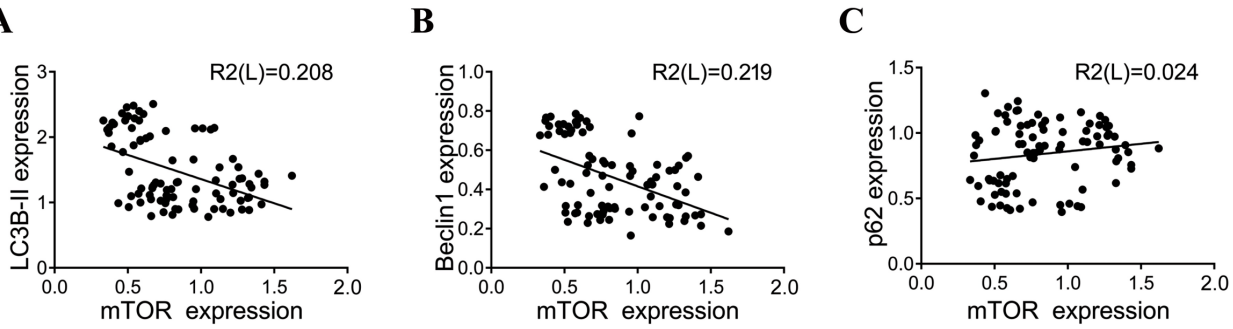

Figure 7 – ***Correlation between expressions of mTOR and autophagy-related proteins in term human placentas: (A) Correlation between mTOR and LC3B-II, n=90, r=-0.456, p<0.01; (B) Correlation between mTOR and Beclin1, n=90, r=-0.468, p<0.01; (C) Correlation between mTOR and p62, n=90, r=0.156, p=0.143>0.05. Pearson's correlation test. mTOR: Mammalian target of rapamycin.***

Discussion

To date, LC3B-II is a well-characterized protein that is specifically localized to autophagic structures throughout the process from phagophore to lysosomal degradation. Thus, LC3B-II is essential for autophagic activity [28]. In addition, Beclin1, a key signaling component required for autophagosome formation, and p62 that functions as a selective autophagy receptor for degradation of ubiquitinated substrates, are also used as markers of autophagy [24, 29]. Therefore, in this study, we examined the expression of LC3B-II, Beclin1 and p62 for autophagic activity. Our data suggested that placentas with FGR exhibited higher autophagic activity and reduced mTOR level in comparison with placentas of AGA. In pregnancies with fetal overgrowth, placental mTOR expression is up-regulated compared with that of the AGA pregnancies. More importantly, according to the Pearson's correlation test, there was a significantly inverse correlation between the expression of mTOR and LC3B-II, as well as between mTOR and Beclin1 expression in term human placentas. Together, these results indicated that placental autophagy and mTOR might be associated with pathophysiology of FGR. Furthermore, there might be a reciprocal relationship between mTOR and autophagy in human placentas.

Unfavorable *in utero* environment in FGR pregnancies

As is reported, fetal growth requires various substrates, such as oxygen, glucose, and amino acids [30]. During gestation, when fetal nutrient demand cannot be met, FGR develops [31]. The placenta, being an interface between the mother and the fetus, promotes the exchange of nutrients and waste products between the maternal and fetal circulatory systems. Therefore, placental dysfunction has been widely recognized a major cause of FGR. A previous research by Chen *et al.* has proved that the number of capillaries per villous cross-section was significantly lower in the FGR placentas (25–41 gestational weeks, *n*=9) than in the control subjects [32]. In the present study, we demonstrate that capillary volume density of peripheral villi in term human placentas of FGR was declined, compared with that in the control, revealing hypovascularization of peripheral villi in the FGR placentas. Moreover, intraplacental vascular flow in FGR pregnancies is difficult or impossible to evidence by color Doppler ultrasound, which indicated inadequate uteroplacental perfusion and reduced placental substrate transport capacity [33]. Secondary to placental malperfusion, a repetitive hypoxia-reperfusion type of injury may occur, resulting in oxidative stress, which can compromise normal function of cellular components including mitochondria and the endoplasmic reticulum (ER), leading to disturbances in energy metabolism and placental protein synthesis [34, 35]. All these result in a hypoxic, nutrient deprived *in utero* environment in FGR pregnancies.

Autophagy in human placentas

Up to now, autophagy has been increasingly identified as a process of cellular self-digestion in infections, cancer, aging and neuronal diseases [17]. However, its role in placental development and fetal growth has not yet been clarified. Herein, we found that autophagic vacuoles were more abundant in the FGR placentas than the control subjects, including trophoblasts, endothelial cells and stromal cells, respectively. Syncytiotrophoblasts, the placental barrier cells that cover the placental villi, enables gas and nutrient exchange between maternal and fetal blood circulation. A dynamic model of uteroplacental blood flow in FGR developed by Roth *et al.* [36] indicates that the wall shear stress is elevated in central parts of the intervillous space (IVS). As is reported, high wall shear stress could damage the syncytiotrophoblasts or at least challenge its cellular turnover [37, 38]. Recently, Liu *et al.* [39] proved that shear stress induced by laminar flow could promote autophagy in human vascular endothelial cells. The flow-conditioned cells are more resistant to oxidant-induced cell injury. Once autophagy is inhibited, this cytoprotective effect is abolished. Therefore, the increased autophagic vacuoles in the trophoblasts of FGR placentas is likely to result from the elevated shear stress in the IVS, playing a cytoprotective role.

To the best of the authors' knowledge, the flow velocity and wall shear stress in the villi capillaries of FGR pregnancies have not been directly measured. It has been previously suggested that mechanical factors could promote proliferation of villous endothelial cells [40]. Furthermore, shear stress-induced autophagy in human vascular endothelial cells provides cytoprotection from oxidant injury [39]. Hence, we believe that the most probable cause of the accumulation of autophagic vacuoles in villous endothelial cells derived from FGR placentas is mechanical factors. The enhanced autophagy may protect endothelial cells against oxidant damage.

Placental mesenchymal stromal cells (MSCs) are the predominant cellular component in human placentas, functioning as structural support for trophoblast villi and vascular network [41]. Based on inadequate uteroplacental perfusion and hypovascularity of peripheral villi, FGR placenta-derived MSCs (PDMSCs) are more likely to suffer from hypoxia. A recent study demonstrated that hypoxia could promote autophagy of human placental chorionic plate-derived MSCs [42]. In addition, Mandò *et al.* reported decreased endothelial differentiation of FGR-PDMSCs [43]. It has been proved that activating autophagic pathway could inhibit endothelial differentiation of bone marrow-derived MSCs [44]. Thus, the accumulated autophagic vacuoles in the mesenchymal stromal cells derived from FGR placentas, is likely a result of hypoxia, leading to poor endothelial differentiation.

In the present study, the increased levels of LC3B-II and Beclin1 expression in the FGR placentas, which was consistent with the previous study [45], revealed enhanced activation of autophagy in FGR pregnancies. In addition, p62, a selective substrate for autophagy, was attenuated in the placentas with FGR, implying increased autophagic degradation of proteins in FGR pregnancies. *In vivo* and *in vitro* studies have demonstrated that autophagy can be induced by nutrient starvation [46, 47]. Incubating neonatal rat cardiomyocytes in serum-free media resulted in elevated autophagic flux and accumulation of LC3B-II and Beclin1. Besides, inhibition of autophagy by 3-methyladenine enhanced cell death, rather than diminished it [46]. These results suggest that autophagy plays a prosurvival role against nutrient deprivation in mammalian cells. Hypoxia is a crucial factor in the promotion of autophagy in human trophoblasts [20, 21]. As reported by Nakashima *et al.* [20] and Saito & Nakashima [21], soluble endoglin (sENG), a preeclampsia-related substance, inhibited autophagy in extravillous trophoblast cells (EVTs) under hypoxia, resulting in poor EVTs invasion and vascular remodeling. Supplementation of adenosine triphosphate (ATP) under hypoxic conditions could rescue the impairment of EVTs invasion [20, 21]. Additionally, the invasion and vascular remodeling under hypoxia were significantly reduced in autophagy-deficient EVTs compared with autophagy-normal EVTs [20], suggesting that autophagy, as a crucial energy provider, had a potential protective role in EVTs invasion and vascular remodeling. As mentioned before, inadequate uteroplacental perfusion and hypovascularization of placental villi in FGR pregnancies contribute to a hypoxic and nutrient deprived intrauterine environment [32]. Hence, we consider that the enhancement of autophagy in FGR placentas is likely a result of adverse *in utero* environmental stimuli, playing a protective role in trophoblast invasion and vascular remodeling, resisting placental insufficiency.

mTOR linked to placental metabolism and amino acid transport in FGR pregnancies

The present study showed that total mTOR protein level was reduced in placentas with FGR *versus* AGA. In the past decades, alterations in placental metabolism and amino acid transport have been described in FGR placentas [48–50]. There were a number of metabolites significantly different between FGR and controls placental explants cultured at different O_2 tensions [50]. In addition, a reduction in the activity of transporters for essential amino acids was observed in human placentas with FGR [48, 49]. It is thereby implying an important role of placental metabolism and amino acid transport in the pathophysiology of FGR. As reported by Kim *et al.* [13], arginine, leucine, and glutamine acted coordinately to stimulate proliferation of primary porcine trophectoderm cells through activation of mTOR signal transduction pathway. Besides, angiopoietin-2, a growth factor highly enriched in placental tissue, and glucose, a major nutrient, induced trophoblast cells proliferation through mTOR signaling [12]. Therefore, we believed that the down-regulation of placental mTOR in FGR pregnancies is likely to result from the widespread changes in the placental metabolism and altered amino acid transport, and it may give rise to abnormal proliferation of trophoblasts.

The relation between mTOR and autophagy

In mammalian cells, mTOR functions as an inhibitor of the initiation step of autophagy by preventing activation of the mammalian autophagy-initiating kinase ULK1 [23]. However, few studies have addressed the relationship between autophagy and mTOR in human placentas. According to the *in vitro* study by Chen *et al.* [51], coexposure of trophoblasts to rapamycin plus bafilomycin resulted in increased LC3B-II levels, compared with exposure to bafilomycin alone, suggesting that inhibition of mTOR induced autophagy in trophoblast cells. Also, Choi *et al.* [52] demonstrated that expression of phospho-mTOR (p-mTOR) and class III phosphatidylinositol-3 kinase (PI3K-III) were significantly reduced, and LC3B-II level and formation of autophagosome were augmented in the trophoblast cells cultured under hypoxic conditions. These results indicated that mTOR is likely to play an inhibitory role in regulation of autophagy in trophoblast cells. However, a recent report by Bernard *et al.* [53] showed that, in fibroblasts, sustained autophagy was associated with inhibition of mTORC1 (mammalian target of rapamycin complex 1) activation and, unexpectedly, enhanced mTORC2 activation, which in turn triggered connective tissue growth factor (CTGF) dependent myofibroblast differentiation. This finding raises the possibility that autophagy may be a novel activator of mTORC2-signaling in human placentas.

Conclusions

Down-regulation of mTOR in FGR placentas potentially due to changes in placental metabolism and substrate transport is associated with abnormal placental function. Proper autophagy in response to unfavorable intrauterine environment including oxidative stress and nutrients deficiency may play a protective role against placental insufficiency in FGR pregnancies. There may be a reciprocal regulation between mTOR and autophagy in human placentas with FGR. Further research on the precise mechanism of crosstalk between mTOR and autophagy is needed, which is an essential step in highlighting potential therapeutic options for suboptimal fetal outcome from pregnancies characterized by placental insufficiency.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author
Weiwei Song, Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, 36 Sanhao Street, 110004 Shenyang, China; Phone +86 13909822679, e-mail: songww1970@163.com

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