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Altered expression of mTOR and Autophagy in term normal human placentas

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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). LC3B 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 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 (FGR)

Introduction
Abnormal birth weight is associated with a variety of diseases, including type 2 diabetes mellitus, hypertension, hyperlipidemia and cardiovascular disease [1, 2]. 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].

In regard to 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 has 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. 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 10th and 90th 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.

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 kept at -70°C for further processing.

Electron microscopy

Placental samples were cut into 2-mm pieces within 10 mins of delivery and placed into fixative (2% glutaraldehyde, 0.1 M sodium cacodylate), stored at 4°C for 24 h. Afterwards, tissues were processed into thin sections. A FEI Tecnai 20 Transmission Electron Microscope (FEI North America NanoPort) was used to capture images.

Immunohistochemistry

Sections of paraffin-embedded tissues were deparaffinized with xylene 3 times for 10 min and rehydrated with a graded series of ethanol washes. Antigen retrieval was achieved by microwaving in 10 mM citrate buffer (pH 6.0), for 30min at 95°C. 3% H2O2 for blocking endogenous peroxidase activity followed by 10% normal goat serum for blocking non-specific antibody binding were used for 30 min at 37°C. Afterwards, the sections were incubated overnight at 4°C, with primary antibodies against human LC3B (Novusbio, NB100-2220, 1:500), Beclin1 (Novusbio, NB500-249, 1:400), p62 (Proteintech, 18420-1-AP, 1:500), mTOR (Abcam, ab2732, 1:800). Slides with absence of primary antibodies were considered as negative controls. After that, slides were probed with biotin-conjugated goat anti-rabbit secondary antibodies (Dako, 1:200) for 40min at 37°C, followed by incubation with avidin-peroxidase (5 μg/ml). Concentrated 3,3-diaminobenzidine (Sigma-fast, Sigma) was then applied to observe immunostaining.

Slides were counterstained with Harris’s haematoxylin and were viewed on a light microscope (OLYMPUS, CX41).

Staining was scored from 0 to 3 by two individuals who were blinded to fetal outcome, with substantial inter-observer agreement (kappa coefficient = 0.81). For scoring, intensity of staining along with the syncytiotrophoblast was taken into consideration: intense, moderate, very weak and absent cytoplasmic staining were considered score 3, 2, 1, 0, respectively. Five microscopic fields at magnification 40x were observed for each specimen.

Western blotting

Proteins were extracted by using RIPA lysis and extraction buffer (Thermo, 89900) and quantified with the Pierce BCA Protein Assay Kit (Thermo, 23227). The 14000× g supernatant from homogenized samples was diluted with loading buffer, boiled for 5 min, and stored at -20°C. 50μg of each protein extract was separated by 12% or 6% SDS-PAGE (depending on the molecular weight of the markers studied) and electroblotted on PVDF membrane (Bio-Rad, Hercules, CA, USA). The PVDF membranes were incubated in 5% skim milk for 1 h 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 GAPDH (Cell Signaling Technology, 2118S, 1:1000) at 4°C overnight. Following incubation with horseradish-peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibody for 2 h at room temperature, bands were revealed by chemiluminescence, and band intensities were quantified using ImageJ software.

Statistical analysis

Data are expressed as mean ± SD, and analyzed by using the Kruskal-Wallis test followed by the Mann-Whitney U test, one-way ANOVA with Bonferroni 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 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).

**Evidence of placental autophagy**

Examination of transmission electron microscopy revealed that autophagic vacuoles were predominantly located in the trophoblast layer of human placentas associated with altered fetal growth (Figure 1, A, B and C). In particular, these autophagic vacuoles were more prominent in the placentas with FGR, containing lysosomal enzymes and semi-digested cell organelles (Figure 1A).

**Expression of placental autophagy**

The main results of immunohistochemistry and western blotting for autophagy-related proteins were concluded as follows: Immunostaining of LC3B and Beclin1 was noted in the cytoplasm of trophoblasts, while p62 was mainly expressed in the cytoplasm and nucleus of trophoblasts (Figure 2, A, B and C). Evaluations of the intensity of the autophagy-related proteins staining (the Kruskal-Wallis test followed by the Mann-Whitney U test) showed a significant increase of LC3B (p<0.001) and Beclin1 (p<0.001), as well as an obvious decrease of p62 (p<0.01) in the FGR placentas compared to the AGA group (Figure 2, D, E and F). Western blotting analysis (one-way ANOVA with Bonferroni post hoc test) further identified that both LC3B-II and Beclin1 expression were significantly up-regulated by 99% and 83%, respectively (p<0.001, Figure 3, A, B and C), and p62 exhibited a 39% decline in the placentas of FGR versus AGA (p<0.001, Figure 3, A and D). Whereas, expression of LC3B-II, Beclin1 and p62 had no significant differences between the LGA and the control group (Figure 3, A-D).

**Expression of placental mTOR**

mTOR was predominantly detected in the cytoplasm and nucleus of the syncytiotrophoblasts from pregnancies associated with altered fetal growth (Figure 4A). Evaluation results of the intensity of mTOR staining (the Kruskal-Wallis test followed by the Mann-Whitney U test) demonstrated that mTOR expression in the human term placentas increased with fetal birth weight (Figure 4B). Immunoblot analysis of mTOR (one-way ANOVA with Bonferroni post hoc test) further confirmed the results of the staining evaluation. In comparison to the AGA group, placental mTOR expression was significantly reduced by 30% in the FGR pregnancies (p<0.01), and raised by 26% in the LGA pregnancies (p<0.05, Figure 4, 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 5A), and also inversely correlated to Beclin1 expression in term human placentas (r = -0.468, p<0.01, Figure 5B). However, there was no obvious correlation between placental mTOR and p62 expression (r = 0.156, p>0.05, Figure 5C).

**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 [25]. 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, 26]. 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 [27]. During gestation, when fetal nutrient demand cannot be met, FGR develops [28]. 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 found highly reduced peripheral villous vascularity in FGR placentas [29]. 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 [30]. 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 [31, 32]. All these result in a hypoxic, nutrient deprived in utero environment in pregnancies complicated by FGR.

**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. Many investigators have reported enhanced trophoblast autophagy in placentas with preeclampsia (PE) [18, 20, 22]. PE is a common hypertensive disorder of pregnancy with a significant impact on maternal and neonatal
mortality and morbidity. Poor placentation and placental dysfunction are implicated in the etiology of this disorder. As described by Oh et al. [18], LC3B-II expression was increased in the placentas with severe PE compared to patients with normal pregnancies. However, expression of placental Beclin1 was not different between normal and PE pregnancies. In early placentation tissues, which suffer from physiological hypoxia, MAP1LC3B puncta were not observed in extravillous trophoblasts (ETVs) at a shallower site from the fetus at the implantation site, but were clearly observed at a deeper site from the fetus side and around the spiral arteries in the implantation site [20]. It is thereby indicated that autophagy may be involved in the pathophysiology of preeclampsia.

In the present study, the increased level of LC3B-II and Beclin1 expression, which was consistent with the previous study [33], revealed that activation of autophagy was enhanced in placentas with FGR. In addition, p62, a selective substrate for autophagy, was attenuated in placentas from FGR pregnancies, 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 [34, 35]. Incubating neonatal rat cardiomyocytes in serum-free media resulted in enhanced autophagic flux and accumulation of LC3B-II and Beclin1. Besides, inhibition of autophagy using 3-methyladenine enhanced cell death, rather than diminished it [34]. These results suggest that autophagy, as an energy provider, is a prosurvival response to nutrient deprivation in mammalian cells. It was reported that, in HTR8/SVneo cells, number of MAP1LC3B puncta was significantly increased under hypoxia as well as starvation compared with that under normoxia, suggesting that hypoxia is a crucial factor in the promotion of autophagy in human trophoblasts [20, 21]. As mentioned before, placentas of FGR have reduced vasculature, contributing to a hypoxic and nutrient deprived intrauterine environment [29]. Hence, we considered enhancement of autophagy in FGR placentas is likely a result of adverse in utero environmental stimuli.

**Dual roles of autophagy in trophoblast functions**

In vitro studies have elucidated that autophagy is indispensable for biological behavior of trophoblasts [20-22]. Gao et al. [22] discovered that, in human trophoblast cell line HTR8/SVneo and human umbilical vein endothelial cells (HUVECs) treated with glucose oxidase, trophoblasts invasion and endothelial cell tube formation were significantly reduced along with accumulated autophagosome and up-regulated expression of LC3B-II and Beclin1. Moreover, inhibition of hypoxia inducible factor (HIF)-1α enhanced trophoblasts autophagy and reduced trophoblasts invasion in conditions of both normoxia and hypoxia [36]. It can be concluded that autophagy may induce failure of trophoblast invasion and vasculature. Paradoxically, soluble endoglin (sENG), a preeclampsia-related substance, inhibited autophagy in EVTs under hypoxia, resulting in poor EVTs invasion and vascular remodeling. While, supplementation of ATP under hypoxic conditions rescued the impairment of cell 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 under hypoxia. Altogether, autophagy probably plays paradoxical roles in trophoblast invasion and vasculature. We believed that autophagy in placentas with FGR, resulting from unfavorable intrauterine environment, may play a protective role rather than a destructive 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 [37-39]. There were a number of metabolites significantly different between FGR and controls placental explants cultured at different O2 tensions [39]. In addition, a reduction in the activity of transporters for essential amino acids was observed in human placentas with FGR [37, 38]. It is thereby suggesting 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 trophoderm 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 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. [40], 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 and coworkers [36] demonstrated that expression of p-mTOR and PI3KIII were significantly reduced, and LC3B-II level and formation of autophagosome were augmented in the trophoblast cells cultured under hypoxic conditions. These results indicate that mTOR is likely to play an inhibitory role in regulation of autophagy in trophoblast cells. However, a recent report by Bernard et al. [41] showed that, in fibroblasts, sustained autophagy was associated with inhibition of MTORC1 activation and, unexpectedly, enhanced MTORC2 activation, which in turn triggered CTGF dependent myofibroblast differentiation. This finding raises the possibility that autophagy may be a novel activator of mTORC2-signaling in human placentas.
Conclusions
In conclusion, down-regulation of mTOR in FGR placentas potentially due to changes in placental metabolism and substrate transport, is associated with abnormal placentation 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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References
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<td>748±102</td>
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Table 1. BMI, body mass index; AGA, appropriate for gestational age; FGR, fetal growth restriction; LGA, large for gestational age. Data are presented as mean ± SD. ***P<0.001 versus AGA.
Altered expression of mTOR and Autophagy in term normal human placentas

Figure 1 – Presence of autophagic vacuoles (arrowheads) in human placentas with FGR (A), AGA (B) and LGA (C) fetus, scale bar = 1µm.

Figure 2 – Immunohistochemical expression of LC3B (A), Beclin1 (B) and p62 (C) in human placentas associated with fetal growth, ×400, scale bar = 50µm. Results of evaluation of the intensity of LC3B (D), Beclin1 (E) and p62 (F) staining were shown. n=30, respectively; **p<0.01, ***p<0.001

Figure 3 – 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, respectively; ***p<0.001.

Figure 4 – Expression of mTOR in human placentas associated with altered fetal growth. (A) Immunohistochemical staining of mTOR, ×400, scale bar = 50µm. (B) Results of evaluation of the intensity of mTOR staining. n=30, respectively. (C) Western blotting showing distinct bands corresponding to mTOR and GAPDH. (D) Quantification of the results in C. n=30, respectively; *p<0.05, **p<0.01.

Figure 5 – Correlation between expression 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.

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