

## Nutrient starvation affects expression of LC3 family at the feto-maternal interface during murine placentation

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**ABSTRACT.** LC3 – the mammalian homolog of Atg8 – was found as autophagosome membrane binding protein in mammals and widely used as an autophagosomal marker. LC3A, B and C show different expression patterns in each tissue. The aim of this study was to reveal the differences of expression patterns among LC3 families in mouse placenta under normal condition and nutrient starving condition. LC3A and B were highly expressed in decidual cells. LC3A and B were increased in D14 compared with D12 and D16 in mouse placenta, while LC3C was decreased. Starvation induced increase in LC3B expression specifically. Immunohistochemistry showed different expression patterns among LC3A, B and C. LC3A expression in syncytiotrophoblast was vanished by starvation. The results of real time RT-PCR suggested differences between D12 and D16 in autophagic cascade induced by starvation. Taken together, this study suggests that autophagy could play a role in placental invasion system and that nutrient starvation affects LC3B expression.

**KEY WORDS:** autophagy, LC3, mTOR, placenta, starvation

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For mammalian reproductive strategy, placental formation is the most important event. Placenta grows rapidly in limited period to maintain the fetus growth. Placental growth occurs by trophoblast invasion into uterine endometrium. By morphological and genetical differences, trophoblasts are divided to several cell types (ex: gianttrophoblast, syncytiotrophoblast and spongiotrophoblast) [7]. Although trophoblast invasion resembles tumor invasion in a manner, placental growth stops at the level of adequate size. At the moment, mechanisms of the placental invasion system remain to be fully defined.

p53 is well known as one of the most famous tumor suppresser genes. It was reported about 50% of tumor has p53 mutation [2]. p53 has multiple functions, such as not only tumor suppression, but DNA repair, proliferation, apoptosis and autophagy [5, 6]. p53 is expressed in trophoblast cell [22], which suggests that p53 is important for placental formation. Uterine specific p53 deficient mice show premature decidual senescence and preterm birth, which occurs via mammalian target of rapamycin complex 1 (mTORC1) signal [13].

Although macroautophagy (simply referred to as autophagy hereafter) has a main role in cytoplasmic turnover,

it is needed for degradation of cytosol and organelle under the starving condition [32]. mTORC1 is important for autophagic modulation against the starvation. mTORC1 is a kinase activated by nutrient signal like amino acid and works for autophagic suppression under the nutrition rich condition [37]. The P70S6K is a key regulator of translation, and it is the most well characterized target of mTOR [3]. P70S6K is well used as an indicator of mTOR. Under the starving condition, p53 induces autophagy via mTORC1 suppression [8]. As negative regulators of mTOR, LKB1 and AMPK are known. On the other hand, PI3K and AKT work as positive regulators of mTOR. Apart from mTOR, p53 and DRAM can induce autophagy directly [8].

Although nutrient starvation induces autophagy in several murine tissues [24], autophagy in the placenta under the starving condition is still unknown. Microtubule-associated protein 1 light chain 3 (LC3) – the mammalian homolog of Atg8 – is widely used as an index of autophagy. LC3 was found as autophagosome membrane binding protein in mammals. LC3 is widely used as a standard marker due to its stability [18]. As human orthologs of Rat LC3, LC3A, LC3B and LC3C were identified. These three orthologs exhibit distinct expression patterns and differ in their post-translation modifications. Although LC3A and LC3C are produced by the proteolytic cleavage, LC3B does not undergo C-terminal cleavage [12]. It has been suggested that these isoforms have redundant and/or distinct roles in each tissue and cell.

In this study, we addressed autophagy – inducing pathway and the cell differences of the LC3 family expression in murine placentas under normal condition and nutrient starving condition.

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## MATERIALS AND METHODS

**Animal:** ICR mice obtained from Kyudo Co. (Saga, Japan) were used in this study. Mice were housed in standard polypropylene cages in a temperature-controlled room (22°C) with a 12 hr light, 12 hr dark cycle. Food and water were provided *ad libitum*. Studies were performed according to protocols for animal use, approved by the Yamaguchi University Animal Experimental Guidelines (permission number: 239).

**Sampling:** ICR female mice of 8 weeks old were mated with male mice of the same strain. The day when a vaginal plug was detected was considered day 1 of gestation. Mice were sacrificed on gestational days 12, 14 and 16 (D12, D14 and D16) under avertin anesthesia. The left uterine horn was fixed in 10% buffered formalin for histological analysis. The right uterine horn was removed, and each placenta was separated to labyrinth zone (LZ) and decidual basalis (DB). For the studies of effects of starvation, pregnant mice were deprived of food for 48 hr before sampling. These mice had free access to drinking water.

**Immunohistochemistry:** Sections of 4  $\mu\text{m}$  in thickness were prepared. After that, immunohistochemistry of MAP1LC3A (Q9H492, EPITOMICS, San Francisco, CA, U.S.A.), MAP1LC3B (18725-1-AP, Proteintech, Chicago, IL, U.S.A.) and MAP1LC3C (AP1804a, ABGENT, San Diego, CA, U.S.A.) was performed. Deparaffinized sections for LC3A were autoclaved in citrate buffer solution pH 6.0 for activation. Next, all deparaffinized sections were treated with 0.3%  $\text{H}_2\text{O}_2$ /methanol for 30 min to inactivate intrinsic peroxidase. Sections were preblocked with 10% goat serum (426042, NICHIREI, Tokyo, Japan) for 30 min before being incubated with specific antibodies described above at 4°C overnight. After that, sections were treated with simplestain MAX-PO (R) (424141, NICHIREI) for 30 min. The immunoreactions were visualized with DAB (D006, DOJINDO, Kumamoto, Japan). Nuclei were stained with hematoxylin. Each diluted concentration is as follows: LC3A (1:100), LC3B (1:100) and LC3C (1:100).

**Western blotting:** MAP1LC3A, MAP1LC3B, MAP1LC3C, p53 (sc-6243, Santa Cruz Biotechnology, Santa Cruz, TX, U.S.A.) and P70S6K (bs-3498R, Bioss, Woburn, MA, U.S.A.) volumes of each region were measured by western blotting. Each protein was extracted with Laemmli Sample Buffer (161-0737, Bio-RAD, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Antibodies used for western blotting are the same as used for immunohistochemistry. Total protein samples were resolved by 8 and 15% polyacrylamide gels and transferred to Immobilon-P Membrane (ISEQ00010, Millipore Corporation, Bedford, MA, U.S.A.). Membranes were blocked in PVDF Blocking reagent for Can Get Signal (NYPBR01, TOYOBO, Osaka, Japan) for 1 hr at room temperature and then incubated with primary antibodies at 4°C overnight. Membranes were washed and incubated with appropriate secondary antibodies (Santa Cruz Biotechnology). Actin (sc-1616, Santa Cruz biotechnology) volumes were also measured as a loading control. Primary antibody and secondary antibody were

diluted with Can Get Signal Immunoreaction Enhancer Solution 1 and 2, respectively (NKB-201 and NKB301, TOYOBO). Each diluted concentration is as follows: LC3A (1:1,500), LC3B (1:2,000), LC3C (1:1,000), p53 (1:1,500), P70S6K (1:1,500), actin (1:4,000) and secondary antibody (1:20,000). Bands were visualized by ImmunoStar LD (296-69901, WAKO, Osaka, Japan) in LAS3000 (FUJIFILM, Tokyo, Japan).

**Real-time RT PCR:** Total RNA was prepared from each sample with ISOGEN II (311-07361, NIPPON GENE, Tokyo, Japan). Reverse transcription was performed with ReverTra Ace (TRT-101, TOYOBO). Real-time PCR was performed in triplicate with Power SYBR® Green PCR Master Mix (4267659, Applied Biosystems, Carlsbad, CA, U.S.A.) in the Step One Real-Time PCR Systems (Applied Biosystems) according to the manufacturer's instructions. GAPDH was used as an internal control. Used primers were as follows: DRAM (Forward: TTGGATTGGTGGGATGCAT, Reverse: GCACGGCTAACTCCTGGAAGT), p53 (Forward: TGCATGGACGATCTGTTGCT, Reverse: TTCCTTGGGCCTTCAAAAA), mTOR (Forward: ACAGCCCGTCACAATGCA, Reverse: GCTACCCGAATCAGCTCTTCA), MDM2 (Forward: GGAGGAAACG-CAGGACAAAG, Reverse: CGATGGCATTGAGGAGAGAG), LKB1 (Forward: ACGGCCTGGAATACCTACACA, Reverse: GTTGCCCGGCTTGATGTC), AMPK (Forward: TCGTGCCCGCCCTTT, Reverse: GGTCAGCATGCCCA-CAAAA), AKT1 (Forward: TCACGCCGCTGATCAA, Reverse: CGGCCTCCGCTCACTGT), PI3K (Forward: CGAGGGAGCAGGAACATCA, Reverse: TGCGGCATAGACAGTGAAGT) and GAPDH (Forward: CATGGCCTTCCGTGTTCCCTA, Reverse: GCGGCACGT-CAGATCCA).

**Statistics:** Numbers of used samples were three for each. Statistical analyses were performed using Kruskal-Wallis test and Mann-Whitney U test as appropriate. *P* values equal or less than 0.05 were considered statistically significant.

## RESULTS

First, we investigated expression volumes of p53 and LC3 family during normal placentation by western blotting (Fig. 1). p53 showed no significant differences between LZ and DB among gestational days tested. LCA and B in DB were significantly higher in expression volume than those in LZ, while LC3C showed no differences. LC3A and B tended to increase at D14, whereas LC3C tended to decrease at D14 (LC3A LZ  $P=0.027$ , LC3B LZ  $P=0.066$ , LC3C LZ  $P=0.039$ . Kruskal-Wallis test).

Next, we investigated changes of LC3 family induced by starvation (Fig. 2). Under the starving condition, P70S6K expression was significantly decreased in LZ and DB at all stages. This suggests starvation induced mTOR suppression and caused autophagy. In association with P70S6K changes, LC3B showed significant increase in LZ and DB at all stages (Fig. 2), while LC3A showed significant increase only in DB at D14 (Fig. 2). LC3C was significantly increased at D12 and D14, but significantly decreased at D16 (Fig. 2).

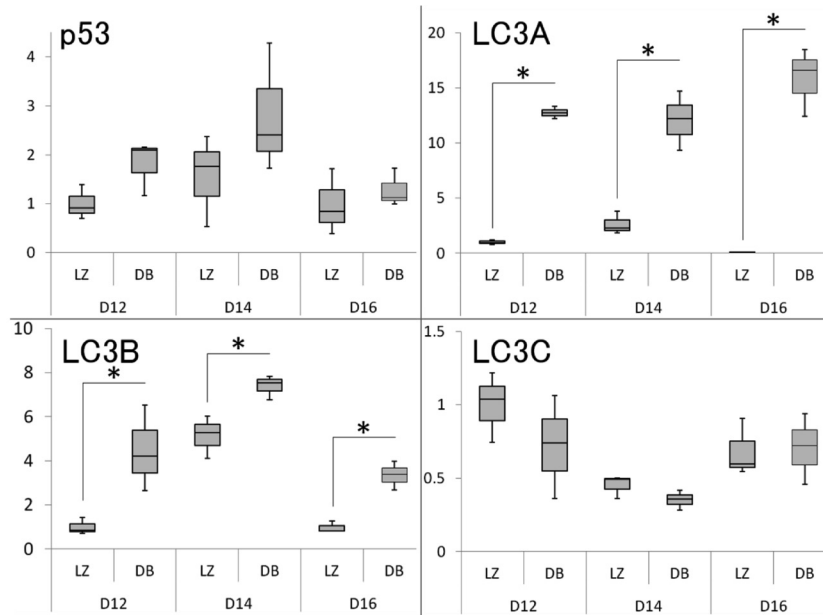


Fig. 1. Western blotting analysis for p53, LC3A, B and C conducted on placental proteins. Each lane represents labyrinth zone (LZ) and decidua basalis (DB) at D12, D14 and D16, respectively. \* $P=0.05$ .

We also observed the expression region of LC3A, B and C by immunohistochemistry. The result is summarized in Fig. 3. While syncytiotrophoblast cells expressed LC3A, B and C, giant trophoblast cells expressed LC3C, but not LC3A. LC3C expressions in spongiotrophoblast cells were positive at D12 and D16, but negative at D14. LC3B was observed in several giant trophoblast cells at D14, but LC3 negative giant trophoblast cells were also observed. LC3A was positive in decidual cells during D12-16. Then, we examined the changes of expression region under the starvation. In syncytiotrophoblast cells, LC3A expression was vanished by starvation, but not LC3B and C (Fig. 4). Other cells did not show remarkable changes (Fig. 3).

Finally, to reveal what signal caused P70S6K decrease and LC3 changes under starvation, we chased changes in the mRNA expression of genes related to glucose starvation and growth factor. The results of real time RT-PCR are shown in Fig. 5. At D12, except for LKB1 in LZ and AKT1 in DB, p53, DRAM, MDM2, LKB1, AMPK, PI3K and AKT1 showed significant increase in starving condition (Fig. 5a-5i). Although there was no significance, LKB1 in LZ and AKT1 in DB also tended to be increased. On the other hand, LKB1 and AMPK showed significant decrease at D16 under starvation (Fig. 5g and 5h).

## DISCUSSION

This study revealed that (i) LC3A and B in DB were expressed higher than those in LZ, (ii) LC3A, B and C expression patterns were different among each cell, (iii) starving induced increase in LC3B expression specifically, and (iv)

glucose starvation and growth factor related genes were changed by starvation during normal placentation. These results suggest autophagy mediated by each LC3 family is functionally distinct.

In normal placenta, LC3A, B and C in LZ at D14 showed differences from D12 and D16. It was reported that HIF-1 $\alpha$  decreases trophoblast invasion via autophagy [4], suggesting that autophagy is related to trophoblast invasion at D14. LC3C decrease at D14 might be the result from compensatory mechanism among LC3 family.

LC3A was strongly expressed in decidual cells. LC3A expression in breast cancer cells is stronger than normal breast cell [27] and important for tumor suppression [1]. These reports suggest LC3A is related to tumor suppression or tumorigenesis. Autophagy works in both tumor suppression and tumor growth [16, 36]. Under the starving condition, a cell induces autophagy, dissolves itself and acquires energy [23]. Although autophagy as a life support system may work for tumor growth, LC3 that reacts strongly against starvation in this study was LC3B, but not LC3A. From this point, LC3A would be related to tumor suppression. LC3A in syncytiotrophoblast was vanished by starvation. This may be occurred for unlocking the autophagic brake of trophoblast invasion to recover the placental growth retardation induced by starvation. Additionally, excessive invasion of trophoblast could be prevented by high LC3A expression in decidual cells during normal placentation.

Starving condition inactivated mTOR, as reflected by reduced P70S6K expression. In association with this, LC3B showed significant increase. Oxygen-glucose deprivation induces LC3B increase in primary cytotrophoblasts [15].

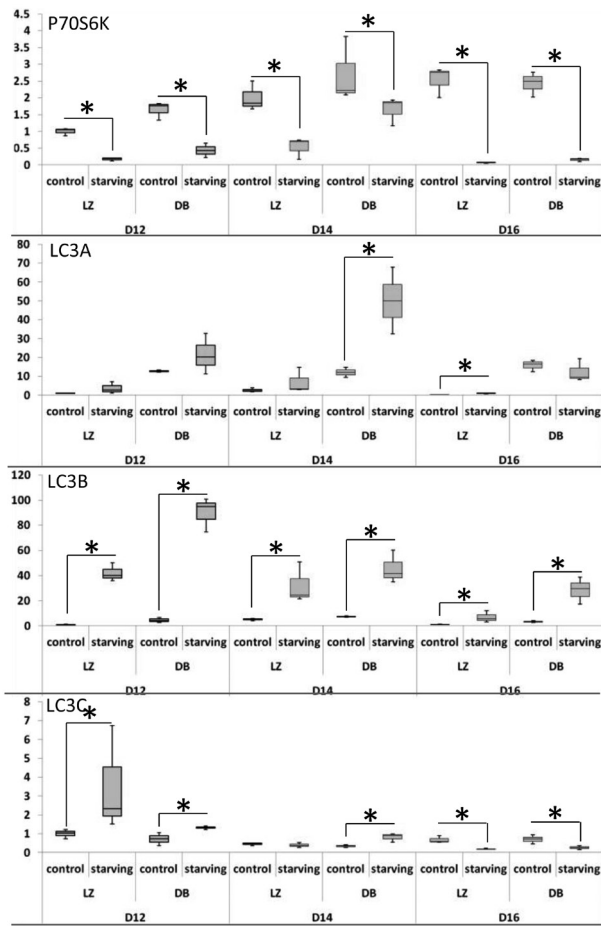


Fig. 2. Western blotting for P70S6K, LC3A, B and C in the normal placenta and placenta under starvation. Each lane represents labyrinth zone (LZ) and decidua basalis (DB) at D12, D14 and D16, respectively. \**P* = 0.05.

Women with intrauterine growth restriction have higher LC3B protein levels than women with normal pregnancy [14]. These reports suggest autophagy induced by starvation occurs via LC3B. Still, LC3B expressions in these reports were related to DRAM increase, but not via mTORC1 suppression. In our study, while DRAM showed significant increase at D12, it had no changes at D14 and D16. Although there is no doubt that LC3B is related to autophagy via DRAM, our results suggest autophagy via mTORC1 is also mediated by LC3B.

LC3C showed significant increase at D12 and D14, whereas it showed significant decrease at D16 under starving condition. The activation of autophagy during infection provides cell-autonomous protection through lysosomal degradation of invading pathogens [29], and there are reports that LC3C is necessary for antibacterial autophagy [20, 34]. Although there are significances, LC3C changes were slight when compared with LC3A and B. LC3C would mainly mediate antibacterial autophagy and would not have an important role for placental growth and starvation. Nonethe-

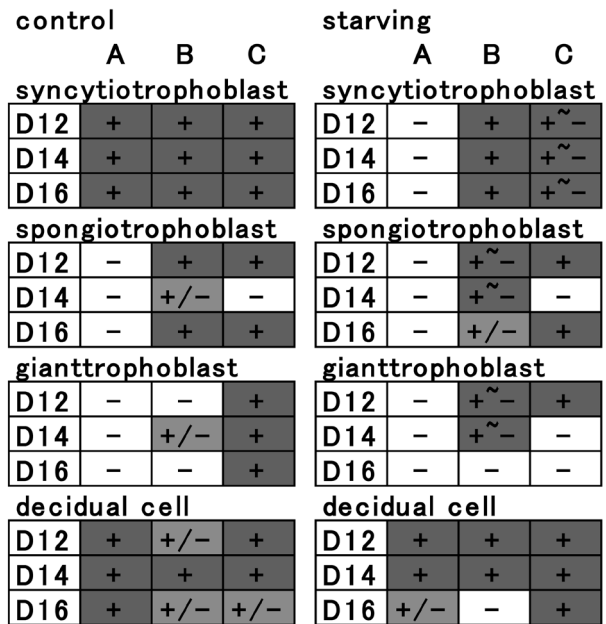


Fig. 3. Summary of immunohistochemistry of LC3A, B and C. Each positive cell and negative cell is indicated by + and -, respectively. +~ indicates weakly stainability compared with + cells. +/- indicates that both positive and negative cells are observed.

less, there is a possibility that LC3C works for compensatory mechanism against other LC3 family.

As signal pathways controlling mTORC1 during starvation, insulin, amino acid and glucose pathways are well known [39]. In this study, starvation induced PI3K and AKT1 increase at D12. Although it suggests this signal is related to mTORC1 expression at D12 under starvation, this signal activates mTORC1 and suppresses autophagy at ordinary times. Nonetheless, considering to P70S6K decreasing, general gene expression seems to move for mTORC1 suppression.

When glucose is exhausted, ATP production is suppressed, and AMPK is activated via LKB1. AMPK phosphorylates TSC2 and suppresses mTORC1 [11]. AMPK also phosphorylates Raptor that is a component of mTORC1 and suppresses mTORC1 [9]. We observed LKB1 and AMPK increase at D12. It suggests that this pathway works at D12 under starvation. AMPK phosphorylates p53 [17], and p53 induces autophagy via DRAM [5]. p53 and DRAM were also increased with LKB1 and AMPK at D12, so glucose starvation - LKB1 - AMPK - p53 - DRAM - autophagy pathway might work at D12.

For the reason of the differences observed at LKB1 and AMPK at D12 and D16, premature placental angiogenesis and insufficient crosstalk at D12 are conceivable. Glucose starvation may not occur at D14 and D16. LKB1 and AMPK decrease observed at D16 could be for the prevention of the excessive autophagy induced by this signal. LC3C decrease at D16 may be related to this signal pathway.

Immunohistochemistry revealed differences in reaction

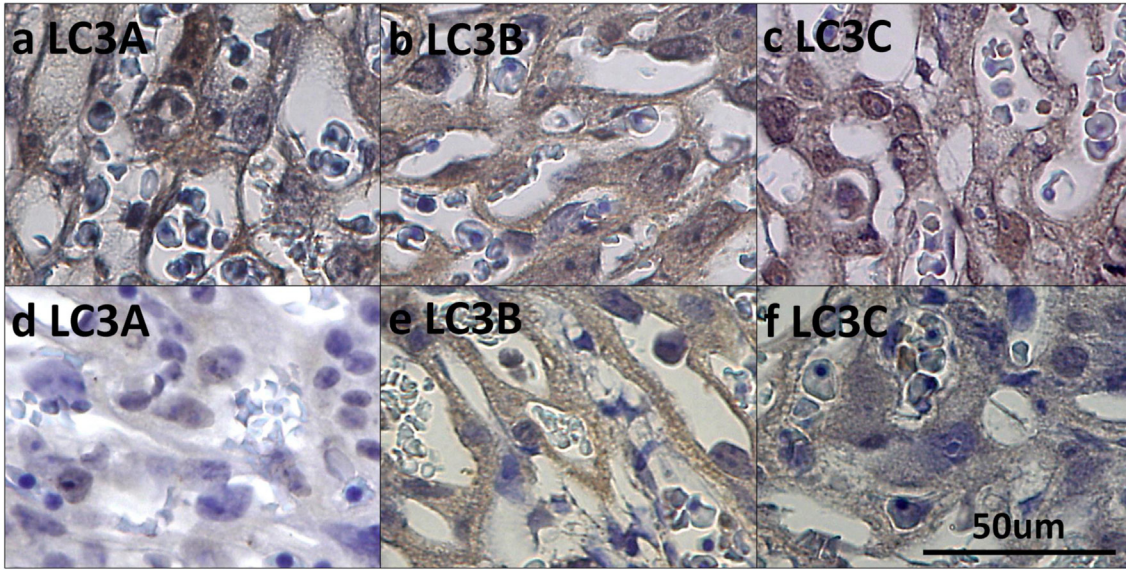


Fig. 4. Immunohistochemical staining of LC3A, B and C. Syncytiotrophoblast cells are shown. a-c shows D14, and d-f shows D14 under starving.

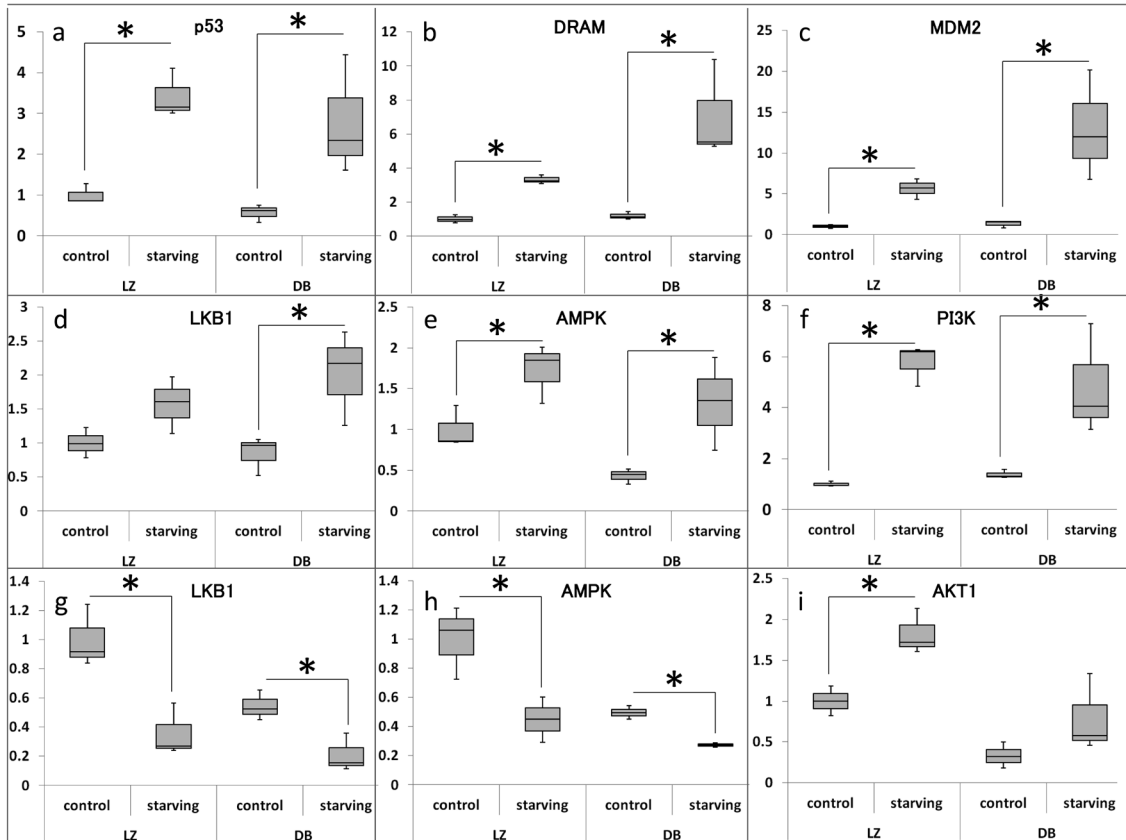


Fig. 5. Real time RT-PCR for p53, DRAM, MDM2, LKB1, AMPK, PI3K and AKT1 conducted in normal placenta and placenta under starvation. a-f, i: D12. g,h: D16. \*P=0.05.

among LC3 family. We suspect these differences were the result from each cell metabolism, function and cell cycle. Nutrients starvation could not affect localization of LC3 family expression, except for LC3A in syncytiotrophoblast. This result suggests syncytiotrophoblast could be affected by starvation.

Autophagy has roles not only in starvation response, but also in keeping quality of protein and organelle, development, differentiation, antigen presentation, tumorigenesis, senescence and more [10, 21, 25, 26, 30, 32, 33, 38]. Additionally, as human Atg8 homolog, there are GABARAP, GABARAPL1 and GABARAPL2 besides LC3 family [19, 28, 31, 35]. Considering from a great deal of variety, it is not strange that these are participating in difference of autophagy. In this study, we observed only LC3A, B and C. GABARAP family may have roles for other function and/or pathway and redundant roles. To clarify all varieties of autophagy, further researches will be required.

In conclusion, the present study strongly suggests that autophagy could be related to placental invasion system and that LC3B is the most sensitive to nutrient starvation in the LC3 family.

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