Placenta 158 (2024) 14–22

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

mTOR is an essential gate in adapting the functional response of ovine trophoblast cells under stress-inducing environments

Irene Viola [a], Paolo Accornero [a], Isabella Manenti [a], Silvia Miretti [a], Mario Baratta [b], Paola Toschi [a,*]

[a] Department of Veterinary Sciences, University of Turin, Largo Paolo Braccini 2, Grugliasco, 10095, Italy
[b] Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11a, 43124, Parma, Italy

ARTICLE INFO

Keywords:
Trophoblast
Early pregnancy
Sheep
Autophagy
mTOR
Nutrients

ABSTRACT

Introduction: During the early stage of pregnancy trophoblast cells adapt to adverse uterine environments characterized by oxygen and nutrient deprivation. Autophagy is an intracellular degradation process that aims to promote cell survival in response to stressful conditions. Autophagy activation passes through the mechanistic target of rapamycin (mTOR), also known as a placental nutrient sensor. Here, we tested the hypothesis that ovine trophoblast cells may adapt to a suboptimal environment through an mTOR dependent regulation of cell survival with relevant implications for key placental functionality.
Methods: Primary ovine trophoblast cells subjected to mTOR inhibitor and low-nutrient conditions were used to explore how autophagy affects cellular functionality and expression of solute carriers' genes (SLCs).
Results: Autophagy activation was confirmed both in rapamycin-treated and low-nutrient conditions, through the detection of specific autophagic markers. However, p-mTOR activation seems to be severely modified only following rapamycin treatment whereas 24h of starvation allowed p-mTOR reactivation. Starvation promoted migration compared to normal culture conditions whereas all trophoblast functional activities were decreased in rapamycin treatment. Interestingly in both conditions, the autophagy-activated environment did not affect the progesterone release. mRNA expression of amino acid transporters remains largely undisturbed except for SLC43A2 and SLC38A4 which are downregulated in starved and rapamycin-treated cells, respectively.
Discussion: The study demonstrates that sheep trophoblast cells can adapt to adverse conditions in the early stage of placentation by balancing, in an mTOR dependent manner, nutrient recycling and transport with relevant effects for *in vitro* functional properties, which could potentially impact conceptus development and survival.

1. Introduction

Normal placenta development is essential to guarantee embryo survival both in the peri-implantation period and later fetus organogenesis. From 16 to 23 days of pregnancy in sheep, trophoblast cells continuously adapt to uterine environment changes characterized by low nutrient and oxygen supply due to inadequate placental blood flow [1]. Therefore, the early placenta physiologically develops in an adverse environment indeed trophoblast cells need to activate several intracellular mechanisms to overcome suboptimal conditions [2]. A major cellular metabolic requirement is the ability to adapt to the external environment to obtain sufficient amounts of energy to sustain cell growth and proliferation. Solute carrier (SLC) transporters control fluxes of nutrients across membranes, representing a critical interface between the microenvironment and cellular metabolism [3]. Moreover, when nutrients decrease below a certain threshold, cellular rescue strategies are triggered to limit anabolic and boost catabolic processes through autophagy. This degradation mechanism transforms intracellular components into simple molecules used as a new nutrient source [4]. In this context, the mammalian target of rapamycin (mTOR) represents a central cellular hub that integrates multiple signals to connect the extracellular environment and cellular needs with an adaptative response.

In human and animal reproduction, mTOR is considered a master regulator of trophoblast cell growth and functionality from embryo implantation to complete placentation [5–8]. In particular, the mTOR

* Corresponding author.
E-mail addresses: irene.viola@unito.it (I. Viola), paolo.accornero@unito.it (P. Accornero), isabella.manenti@unito.it (I. Manenti), silvia.miretti@unito.it (S. Miretti), mario.baratta@unipr.it (M. Baratta), paola.toschi@unito.it (P. Toschi).

https://doi.org/10.1016/j.placenta.2024.09.011
Received 14 November 2023; Received in revised form 13 September 2024; Accepted 15 September 2024
Available online 19 September 2024

complex1 (mTORC1) plays a pivotal role in the placental nutrient-sensing network throughout the downstream regulation of nutrient synthesis and transport [9]. mTORC1 modulates SLCs activity, especially amino acids and glucose carriers suggesting that it constitutes an essential gateway for the long-term health of the conceptus [10–12]. As supported by results from IUGR placenta mTOR downregulation is associated with a reduction of system A and L amino acid transporter activity [13–15]. Moreover, investigations conducted on human and rat placenta have demonstrated, both *in vivo* [15,16] and *in vitro* [17,18], that mTOR signalling is a positive regulator of system A (SLC38s family) and L amino acid transporters (SLC7s and SLC43s families), mediating uptake of essential and non-essential amino acids. This role in nutrient homeostasis has also been suggested in sheep based on a temporal and cell-specific profile of SLCs gene expression in peri-implantation conceptus [19].

Under nutrient-rich conditions, mTORC1 activation promotes trophoblast growth and differentiation, whereas in response to nutrient deprivation, trophoblast cells may activate autophagy process through mTOR inhibition [20–22]. Thus, depending on nutrient availability, we sustain that silencing/activating placental mTOR dynamically regulates the equilibrium between cytoplasmic content recycling (autophagy) and extracellular nutrient uptake (SLCs).

Dysfunctions of placenta development lead to many gestational diseases, including early pregnancy loss in ruminants, intrauterine growth restriction, and preeclampsia in humans [23,24]. However, placental growth adapts to safeguard fetal survival, but how trophoblast-impaired activity may be compensated to allow conceptus development is still incompletely described [25,26]. Previous research demonstrated that trophoblast may activate autophagy both in physiological situations for maintaining cellular balance during periods of short-term nutrient and oxygen deprivation, and in compromised pregnancy as a response to different stressors [27–29].

However, although the well-recognized role of mTOR as a placental nutrient sensor and master regulator of autophagy, how it drives trophoblast cellular fate during early placenta development remains largely unexplored. Thus, our work aims to explore how ovine trophoblast cells adapt to a stress-inducing environment in terms of the main cellular functional activities and nutrient recycling and transport through mTOR-dependent mechanisms.

2. Methods

Materials. All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co. The following chemicals were used: Dulbecco Modified Eagle Medium (DMEM; D6546); DMEM/F12 (Gibco, 21331020), penicillin–streptomycin (Gibco, 15140122); fetal bovine serum (FBS; Gibco, 10270106); l-glutamine (G7513); sodium pyruvate (S8636); non-essential amino acids (Gibco,11140035); insulin (I9278); rapamycin (RAPA, Santa Cruz, sc-3504A); chloroquine (C6628); dimethyl sulfoxide (DMSO; D2650); mouse anti-STAT3 (STAT3, 1:10,000, Cell Signalling, 9131S); mouse anti-alpha-tubulin (aTUB, 1:10,000, T5168); rabbit anti-mTOR (mTOR, 1:1000, Cell Signalling, 2983); rabbit anti-phospho-mTOR (pmTOR, 1:1000, Cell Signalling, 5536); mouse anti-BCLN1 (BCLN1, 1:1000, Santa Cruz, sc-48341); rabbit anti-microtubule-associated protein light chain 3B (LC3B, 1:100 or 1:1000, Invitrogen, 46286); 4,6-diamidino-2-phenylindole (DAPI; Thermo Scientific); Alexa-fluor-594 goat-anti-mouse (1:500, Invitrogen, A11032); horseradish peroxidase (HRP)-conjugated secondary antibodies (1:15,000, Immunopure Goat anti-rabbit and anti-mouse IgG, Thermo Fisher Scientific); growth factor-reduced Matrigel Matrix (BD Biosciences, 354230).

Cell culture treatments. The experiments were conducted using ovine primary trophoblast cells (oTCs) previously isolated and characterized in our works [8,30]. oTCs were cultured with a supplemented trophoblast growth medium using as a control (CTR), composed of DMEM/F12 supplemented with 100 UI/L penicillin and 100 μg/mL streptomycin, 10 % FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 4 μg/mL insulin. Cells were incubated at 37 °C in an atmosphere of 5 % CO2, and the culture medium was replaced every 48h until 80 % confluence. Then oTCs were subjected to low-nutrient conditions using a DMEM (STARV), and 100 nM rapamycin (rapa-CTR, rapa-STARV) for 24h. oTCs were used between 2 and 8 passages. All the tests were performed at least three times starting from different cell batches. The experimental design is described in Fig. 1.

Immunochemistry. oTCs were cultured on four-well chamber slides (Nunc Lab-Tek II Chamber Slide system) under CTR, STARV, and RAPA conditions. Cells were fixed with 4 % paraformaldehyde at RT for 10 min, washed twice with Tris-buffered saline (TBS) for 3 min each, and then non-specific antigen sites were blocked with 10 % goat serum at RT for 2h. oTCs were incubated at 4 °C overnight with LC3B antibody. After washing twice with TBS, the secondary antibody Alexa-fluor-594 goat-anti-rabbit was incubated at RT for 1h in the dark and nuclei stained with DAPI for 10 min. The no-primary antibody control was meanwhile processed similarly (Supplemental Fig. 1). Leica SP8 confocal fluorescent microscope (Leica Microsystems) was used to capture pictures at 20X.

Gene analysis. oTCs were seeded in six-well plates (3×10^5 cells/well). Total RNA was extracted with Maxwell RSC simplyRNA tissue kit (Promega). RNA quality and concentration were checked using Nanodrop (Thermo Fisher), and 1 μg was reverse transcribed together with non-reverse transcribed control (no-RT) using iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories) by Bio-Rad Thermocycler (iCycler Thermal Cycler). cDNA was amplified with a CFX Connect real-time PCR detection system (Bio-Rad Laboratories) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). cDNA and no-RT samples were used for gene expression analysis using specific 5′–3′ primer pairs designed to anneal at 58°/62 °C with already proven amplification efficiency (E) previously published in our papers [8,31] or obtained from others [32]. Each run was performed in duplicate under conditions of 40 cycles of 94 °C for 45 s, 58/62 °C for 45 s, and 72 °C for 1 min. The relative expression of each gene was calculated using the comparative threshold cycle method with ribosomal proteins S9 and L32 (RPS9, RPL32) as housekeeping genes. To avoid false-positive signals, dissociation curve analyses and negative controls (no-sample) were performed in each run.

Protein analysis. 90 % confluent oTCs seeded in six-well plates were washed twice with ice-cold PBS and lysed for 10 min on ice in 150 μL fresh buffer solution (Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, and 1 % Triton X-100) by adding Protease Inhibitor Cocktail (1:100), 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride. Samples were collected and centrifuged at 4 °C for 15 min at 15,000g. Supernatants were quantified with DC Protein Assays (Bio-Rad Laboratories). 10 μg of total protein was separated on 7.5 % or 15 % polyacrylamide gels and transferred to 0.2-μm nitrocellulose (mTOR, pmTOR, BCLN1, STAT3) or polyvinylidene difluoride (LC3B, aTUB) blotting membranes (Amersham Protran Premium). Membranes were blocked at RT for 1h in 10 % BSA TBS (TBS-Tween, 10 mM Tris, and 150 mM NaCl, pH 7.4, 0.1 % Tween 20), then incubated overnight at 4 °C with the primary antibodies. After washing 30 min in TBS–Tween, membranes were incubated at RT for 1h with HRP-conjugated secondary antibodies. Then, membranes were washed in TBS–Tween and incubated for 5 min at RT with Clarity Western ECL Substrate (Bio-Rad Laboratories). Proteins were visualized by exposing the membranes to an autoradiographic CL-XPosure Film (Thermo Fisher Scientific); the bands were acquired with an EPSON Perfection V39 scanner.

Functionality tests. oTCs were subjected to different functional tests as previously described in our paper [8]. Briefly, migration and invasion assays were conducted by seeding 50,000 cells in 100 μL of serum- and insulin-free DMEM into the upper chamber of an 8-μm Transwell chamber (Costar, 3422, Corning), whereas 600 μL of selected culture conditions were added into the lower chamber. Invasion activity was

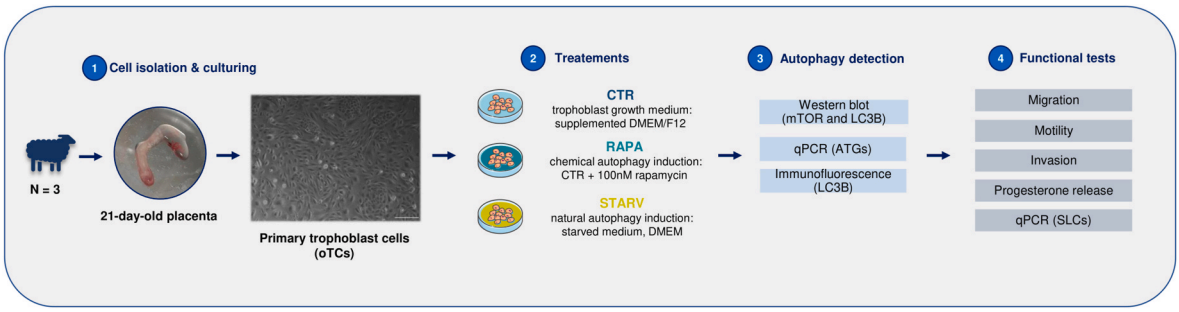


Fig. 1. Experimental design. The figure summarizes the main steps of the study. Cell culturing (step 1): 21-day-old ovine primary trophoblast cells (oTCs) previously isolated and characterized from early sheep placenta were cultured in a trophoblast growth medium at 37 °C in an atmosphere of 5 % CO2 until 80 % confluence. Treatments (step 2): oTCs were subjected to 24 h treatment with 100 nM rapamycin (RAPA) and low-nutrient medium (STARV) to study trophoblast cell adaptation under chemical and natural autophagy induction, respectively. Autophagy detection (step 3): Western blot analysis, qPCR, and immunofluorescence cell staining were performed to explore autophagy-related markers regulation through several stages of the autophagy process, including mTOR modulation, autophagy-related genes expression (ATGs) and autophagosome protein detection (LC3B). Functional tests (step 4): the effect of previous treatments on oTCs behavior was explored through the Transwell method (with or without Matrigel-coated) for cell migration and invasion assessment; wound-healing and ELISA assays were used to study motility and progesterone release, respectively; moreover, the expression of nutrient carrier transporters genes (SLCs) was inspected thanks qPCR analysis.

assessed in the same manner but using Transwell coated with Matrigel. After 12h, the non-migrated cells were removed by washing twice with PBS. The inserts were fixed in 4 % formaldehyde for 15 min, stained with DAPI for 10 min at RT, and washed with PBS. Cells were counted in 25 random non-overlapping fields acquired at a magnification of 10X by using a Leica AF6000 LX (Leica Microsystems) fluorescent microscope.

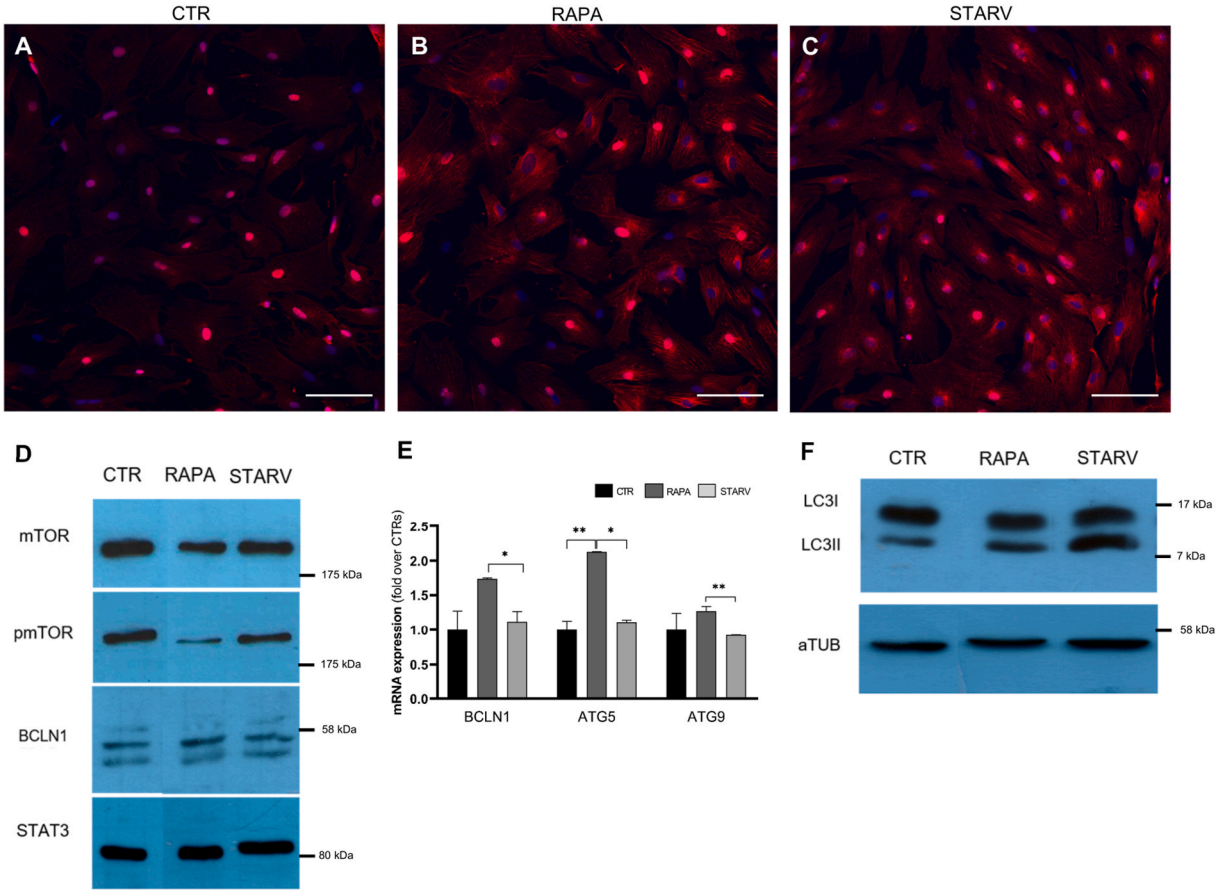


Fig. 2. Autophagy process modulation in oTCs under rapamycin and low-nutrient culture conditions. oTCs were cultured in trophoblast growth medium until 80 % confluence, then starved for 12h and subjected to different treatments (trophoblast growth medium, CTR; 100 nM rapamycin, RAPA; low-nutrient, STARV). After fixing with 4 % paraformaldehyde, non-specific antigen sites were blocked with 10 % goat serum and incubated at 4 °C overnight with LC3B antibody at 1:100 dilution; nuclei were counterstained with DAPI. Pictures were taken using the same confocal microscope parameters at 20X magnification (A, B, C, scale bar: 100 μm). Immunocytochemistry showed a basal level of LC3B in a normal culture trophoblast growth medium compared to rapamycin and nutrient deprivation conditions in which a higher expression was observed. For both ATG genes, mTOR and LC3B expression analysis, RNA and protein were extracted from 24 h oTCs subjected to different culture conditions. The picture shows an immunoblot of mTOR, pmTOR, and STAT3 (loading control) (D); LC3B (I and II), and aTUB (loading control) (F). ATGs expression in oTCs was evaluated through qPCR analysis (E). The Kruskal-Wallis was used, and data were reported as mean ± s.e.m. (*$P < 0.05$; **$P < 0.01$). The experiments were repeated three different times, and each qPCR analysis was duplicated.

oTCs motility was studied through a wound-healing assay. 50,000 oTCs were cultured till 80 % confluence and starved for 12h. A straight line was drawn with a sterile p-200 pipette tip in the center of the dish, removing detached cells by washing twice with PBS. The medium was added following the experimental conditions. Images were captured every 6h with a Leica AF6000 LX and analyzed with ImageJ Fiji 1.53s (National Institutes of Health, USA; https://imagej.nih.gov/ij/) [33]. Finally, progesterone extraction was performed thanks to modifications of the DetectX® Steroid Liquid Sample Extraction protocol (Arbor Assays) as we previously described [8]. Then, hormone concentration was detected using Progesterone ELISA kit (DRG Diagnostics GmbH) and expressed as pg/100,000 cells.

Statistical analysis. For each assay, data were obtained from at least three different replicates. The Kruskal-Wallis one-way analysis of variance by ranks test was performed for cell migration, invasion, motility, progesterone release, and gene and protein expression data. When these multiple comparisons were significant, pairwise comparisons using the nonparametric Mann-Whitney *U* test were conducted. Data are reported as the mean ± s.d. (standard deviation) or mean ± s.e.m. (standard error) (for motility assay, gene, and protein expression). Statistical differences were considered significant when P was <0.05.

3. Results

Autophagy process modulation in starved and rapamycin-treated oTCs. Since autophagy is a multi-step process, we investigated the autophagy regulation on oTCs through multiple approaches, including protein and gene expression profiles of well-known autophagic markers (Fig. 2). Western blot revealed the activation of the autophagy mechanism through the inhibition of mTOR in rapamycin-treated cells, whereas mTOR phosphorylation was detected in starved cells (Fig. 2D; Supplemental Fig. 2). Immunochemistry displayed the presence of LC3B in every condition, with a higher expression under low-nutrient and mTOR-inhibited conditions compared to the control (Fig. 2A, B, C). The induction of autophagy was also explored by quantifying the LC3II/LC3I protein ratio. In a normal environment, oTCs did not show an increase of LC3II as well as in rapamycin-treatment cells; on the contrary, the LC3II protein expression was more evident in starved-oTCs (Fig. 2F; Supplemental Fig. 2). Autophagy activation was also confirmed by BLCN1 protein which is highly expressed in rapamycin and starved oTCs (Supplemental Fig. 2). Finally, autophagy-related genes modulation (BCLN1, ATG5, ATG9) was studied through qPCR (Fig. 2E), and ATG5 expression results increased in rapamycin-treated cells compared to control ($P < 0.01$); while no difference was observed in nutrient-deprived oTCs.

Trophoblast functional activities in starved and rapamycin treatments. Under starved conditions, migration activity severely increased both compared to control and rapamycin-starved treatment ($P < 0.0001$) (Fig. 3A). The motility graph displayed how the two-dimensional cell movement followed a different pathway under the experimental conditions among 36h of culture (Fig. 3C and D). From 0 to 12 h, oTCs closed the scratch gap in the same manner. Only after, cell motility was decreased by rapamycin treatment (18h, $P < 0.001$). In contrast, nutrient-deprived oTCs showed a slightly low activity compared to normal culture conditions (18h, 24h, $P < 0.01$). No difference was observed between the two rapamycin-treated conditions. Then, the ability of oTCs to degrade the extracellular matrix was explored through invasion activity (Fig. 3B, Supplemental Fig. 3). Cells seemed to similarly invade both in the presence of trophoblast growth medium and starved environment. On the contrary, rapamycin treatment decreased trophoblast invasiveness ($P < 0.0001$; $P < 0.05$), without any difference between the rapamycin-control and rapamycin-starved treatment. Moreover, starved and rapamycin conditions did not influence the progesterone release (Fig. 3E).

A panel of SLC genes was identified by selecting target genes involved in system L (Leucine preferring; SLC7A8; SLC43A2) and A (Alanine preferring; SLC38A4) neutral amino acid transporter as well as in ASC (alanine, serine, cysteine preferring; SLC1A4) and N (glutamine preferring; SLC38A7) systems [34,35] (Fig. 4). qPCR analysis showed that mTOR-inhibited environment increased SLC1A4, SLC7A8, SLC38A7 ($P < 0.05$) and SLC43A2 ($P < 0.01$) expression compared to starved-oTCs. SLC38A4 was downregulated in rapamycin-treated cells both compared to control ($P < 0.05$) and starved cells ($P < 0.01$). Generally, gene expression of all selected SLCs remained unchanged between normal growth medium and low-nutrient conditions, except SLC43A2 expressing a decrease in a starved environment ($P < 0.01$).

4. Discussion

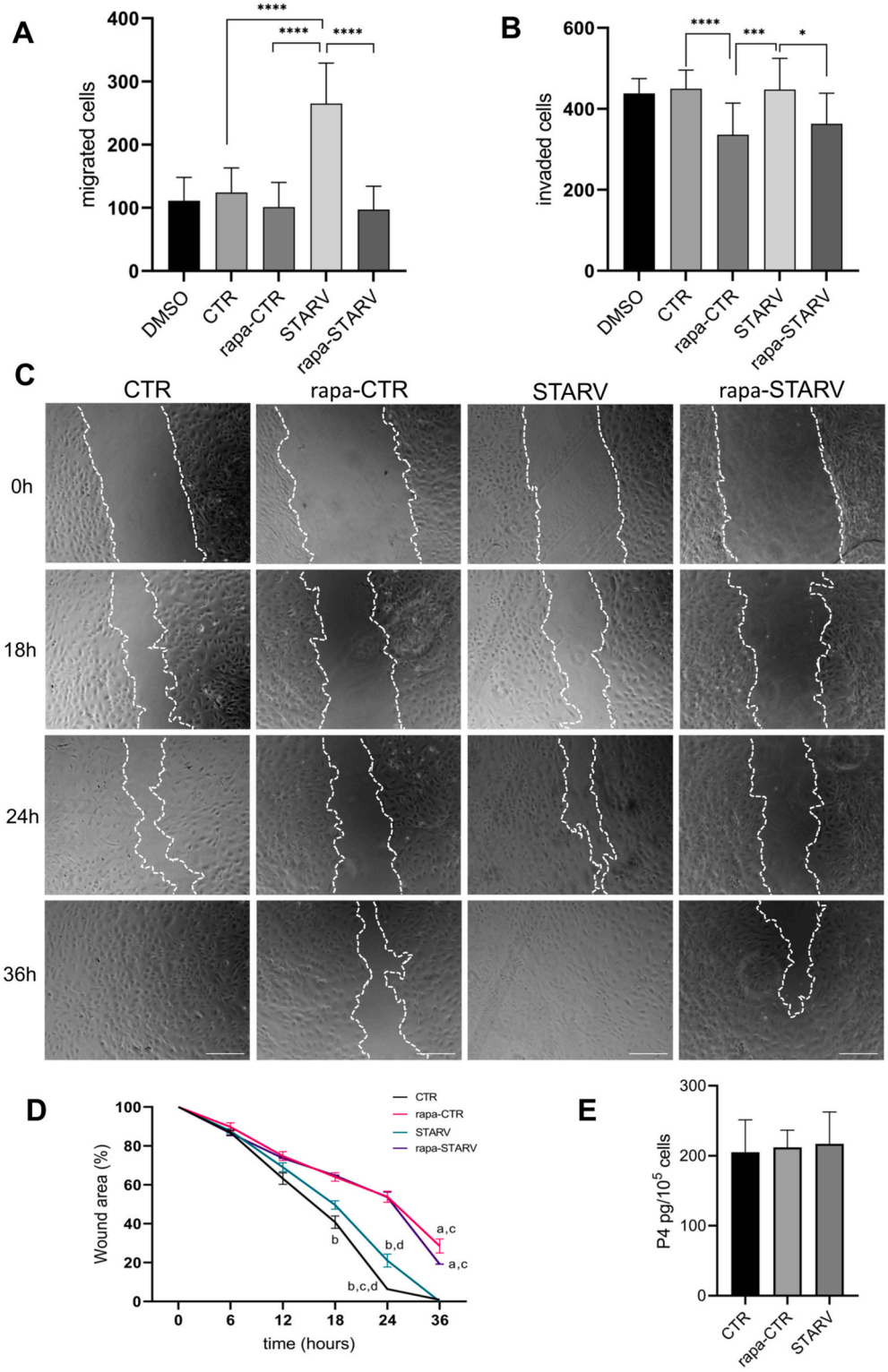
When facing adverse conditions such as low nutrient or oxygen availability cells could adopt different adaptation strategies to survive in the suboptimal environment. These include modulation of typical cellular properties including migration, invasion, and hormone release as well as activation of rescue mechanisms such as autophagy.

In the placenta, mTOR acts as a nutrient sensor network regulating trophoblast cell growth and metabolism [10]. It integrates different upstream extracellular signals, including nutrients, oxygen, and growth factors with downstream effects ranging from modulation of activity of key nutrients transport to activation of the autophagic process. Here, we explored the mTOR-dependent adaptation strategies used by trophoblast cells to survive the adverse uterine environment that inevitably occurs during early pregnancy.

A previously characterized oTCs model was used to study what occurs following mTOR inhibition (Rapamycin) or in the case of nutrient and growth factors deprivation (Starvation). The effects of these stressed conditions were evaluated in terms of changes in cell functional proprieties, autophagy activation, and modulation of nutrient carrier expression.

First, to assess autophagy activation we focused on the main autophagy marker LC3B [4]. Immunoblotting evaluation of the LC3II/LC3I revealed that the amount of LC3II increased in starvation conditions compared to controls while rapamycin-treated cells did not exhibit the expected LC3II/LC3I ratio described in other cellular systems subjected to the same treatment. To exclude a defect in the autophagic mechanism, as suggested by specific guidelines [36], rapamycin-treated cells were exposed to chloroquine which allows autophagy flux visualization by blocking autophagosome degradation (Supplemental Fig. 4). In this way, an increase in LC3II has been detected in rapamycin-treated cells confirming autophagy activation following mTOR inhibition. To further corroborate our data, we decided to repeat the analysis using the oTr cell line (kindly provided by Prof. FW Bazer), a widely validated cellular model used in a large number of sheep placental studies [37]. We have applied to oTr the same stressful condition used for oTCs and we have obtained similar results suggesting that this unusual LC3II/LC3I ratio is a common feature of sheep trophoblast cells (Supplemental Fig. 4). Accordingly, autophagy studies [38,39] conducted on human trophoblast cells reported conflicting results in terms of autophagic response following treatment with canonical activator or inhibitor used in many others *in vitro* cellular systems, leading the authors to conclude that this is a prerogative of trophoblast derived cells [39].

Immunocytochemistry analysis confirmed that trophoblast cells subjected to normal culture conditions exhibited a basal level of LC3B detection, whereas fluorescence intensity increased in rapamycin-treated and starved cells. We observed a diffuse cytoplasmic distribution of LC3B; however, a predominant accumulation of LC3B in perinuclear and nuclear areas has been noticed. Despite the autophagic process occurring in the cytoplasm, the presence of LC3B in the nucleus has been recently reported in a growing number of studies [40,41] and various hypotheses about its role have been proposed. According to some authors, in starvation conditions, it may be used as a reserve for LC3 cytoplasmic pool [42]. For others, it may be involved in the degradation of nuclear lamina [43] or in managing nucleolar activity in



(caption on next page)

Fig. 3. Trophoblast functional activities under low-nutrient and rapamycin culture conditions. The figure shows oTCs migration, motility, invasion activity, and progesterone release under 24h experimental conditions (CTR, trophoblast growth medium; rapa-CTR, CTR + 100 nM rapamycin; STARV, DMEM; rapa-STARV, STARV + 100 nM rapamycin). (A) Migration and (B) invasiveness were explored by the Transwell system, with and without Matrigel-coated. The migrative and invasive activity of 50,000 trophoblast cells were explored thanks to an 8-μm Transwell chamber. Both tests were performed three different times. Results reported (mean ± s.d.) the number of total cells counted after 12 h in 25 random non-overlapping fields. The Kruskal-Wallis was performed (*P < 0.05; **P < 0.01; ***P < 0.001). Starved conditions induced an increase in migration activity compared to both control and rapamycin treatment (A). On the contrary, no difference was observed in invasion activity in starved oTCs compared to control, but a slow activity was again confirmed in the mTOR-inhibited system (B). (C–D) A wound healing assay was carried out on oTCs subjected to the abovementioned culture conditions for 36 h to study cell bi-dimensional motility (C, scale bar: 100 μm). Pictures showed oTCs movement at the starting point, 18, 24, and 36 h (C). The graph describes the extent of gap closure over time (D). The experiment was repeated in triplicate twice. The starved condition seemed to not affect cell movement until 24h of culture compared to the control, while both rapamycin treatments negatively affect oTCs motility, without any differences among them. Letters a, b, c, and d denote differences (P < 0.05) vs CTR, rapa-CTR, STARV, and rapa-STARV, respectively. The Kruskal-Wallis was performed, and data are shown as mean ± s.e.m. (E) Progesterone release (P4) in the culture medium was detected after 24 h treatment. P4 was extracted using the diethyl ether method at −80 °C, then concentration was determined by an enzyme-linked immunosorbent assay kit and expressed as pg/100,000 cells. Analyses were repeated twice on three different endpoint analyses at least. Data expressed as mean ± s.d. suggested no differences between treatments.

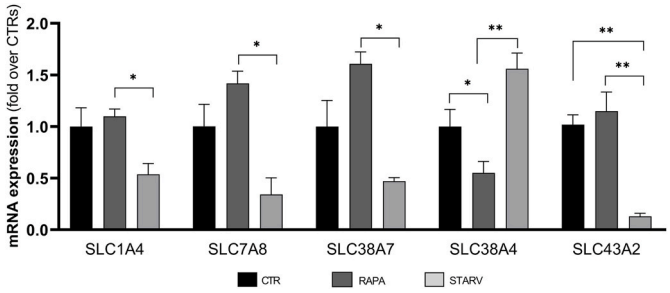


Fig. 4. SLCs expression in oTCs in starved and rapamycin culture environments. SLCs gene expression was defined after 24h 100 nM rapamycin supplementation as well as starved conditions in oTCs. All experiments were performed at three different times at least, and each qPCR analysis was duplicated. The Kruskal-Wallis was performed, and data were reported as mean ± s.e.m.; significance is denoted with an asterisk when *P < 0.05 or **P < 0.01. All SLC genes were upregulated in mTOR-inhibited oTCs, apart from SLC38A4 expressing a significant decrease both compared to control and low-nutrient cells. No difference in solute carriers' gene expression was observed between oTCs cultured in a normal trophoblast growth medium and a nutrient-deprived environment, except for SLC43A2 severely downregulated in starved oTCs.

response to cellular stress [44,45]. Thus, providing a clear interpretation of LC3B nuclear detection is still not possible due to the lack of knowledge about this controversial aspect.

Moreover, protein expression of another autophagy marker (BLCN1) [46] was evaluated and its increase in both suboptimal environments confirms that the placenta evokes this rescue mechanism as a part of its adaptation strategies.

Once established that the applied treatments were able to differently trigger the induction of autophagy we evaluated the consequences for the main trophoblast functional proprieties. Migration, motility, and invasion activities are considered of paramount importance for normal placenta development [47]. After all, from 16 to 23 days of pregnancy, ovine trophoblast cells migrate from trophectoderm tissue for taking first contact with the uterus, and then move onto the maternal epithelium to partially invade the caruncular areas [48,49]. To date, little is known about the migrative and invasive behavior of ruminant trophoblast cells in the early stage of placentation, when an unfavourable event is more likely to occur. This work is the first exploring how ovine trophoblast cells adapt their functional activities to the surrounding environments.

We observed that oTCs responded by inducing migration in low-nutrient conditions, suggesting that starvation represents a "good" stimulus for trophoblast cells' adaptation. This may be because trophoblast biologically lives in a nutrient-poor uterine environment during this developmental period, so the placenta reacts to nutrient deprivation by moving towards the maternal side. However, motility indicateed that oTCs withstand low-nutrient conditions for a short time since the bi-dimensional movement ability decreases at 24h. Nevertheless, our results also displayed that if rapamycin suppressed mTOR, starvation did not stimulate either migration and motility, suggesting a predominant effect of mTOR in trophoblast cells adaptation.

Conflicting findings regarding the role of autophagy in cellular invasion have been described. *In vivo* experiments on food-deprived pregnant mice reported autophagy activation through mTOR/p70 signalling in the early placenta [50]. They have supposed that under starved conditions autophagy-mediated trophoblast activity is shifted to cellular growth rather than invasion. Moreover, in both suppressed-autophagy JEG3 and HTR8/SV cells the invasiveness was increased [46]. Following rapamycin treatment, we observed a decrease in trophoblast cell invasion, whereas nutrient deprivation seemed to not influence it compared to the normal environment. In this view, our findings point out that low-nutrient conditions have a greater impact on migration than on invasive activity. It suggests that when trophoblast cells first contact the endometrium, the nutrient lack is compensated by syncytium formation with the endometrial cells. Regrettably, this assumption cannot be confirmed because we solely focused on trophoblast cells' ability to degrade an extracellular matrix, but without endometrial stimuli.

This study also revealed that oTCs progesterone secretion was not impacted by the stress-inducing treatments, underling that autophagy activation was not involved in the downstream regulation of this hormone production and release. This evidence highlights also that in autophagy-activated status trophoblast preserves some key functional features for survival, in agreement with previous data for hCG in human primary trophoblast subjected to silencing of both mTOR complex [18]. Therefore, we can assert that autophagy activation has much more impact on the initial migration and motility during which trophoblast cells must continuously adapt to adverse environments.

Furthermore, due to the evidence that mTOR inhibition markedly decreases the activity of key placental amino acid transporters [17] and the occurrence of reduced protein expression of specific SLC isoforms in response to a maternal low-protein diet [16], we decided to explore the expression of selected SLC genes, involved in essential and non-essential amino acids transport. Gene expression analysis revealed that SLCs mRNA levels in rapamycin-treated cells resemble the expression profile of the controls except for SLC38A4, which is significantly downregulated following mTOR inhibition. On the other hand, cells grown in starvation displayed a markedly reduced expression of SLC43A2 compared to controls, while other genes remained unaltered. This agrees with previous results showing that in human primary trophoblast cells, following mTORC1 inhibition, the decreased amino acid uptake does not change global SLC protein or mRNA expression [18,51], supporting the hypothesis that mTOR regulates amino acid transporter activity at the post-translational level.

In human primary trophoblast cells, silencing of mTORC1 or mTORC2 decreased basal System A and L transport activity while their simultaneous inhibition completely inhibited amino acid uptake,

suggesting that they independently regulate amino acid transport [18]. This could explain why, although mTOR inhibition, in our rapamycin-treated cells SLCs expression remains largely unaltered. As supported by decreased p-mTOR expression, rapamycin targeting specifically mTORC1 while mTORC2 activity remains undisturbed (as confirmed by our previous results about phospho-Ser-473-Akt expression [8]) and could partially sustain nutrients transporter activity, without markedly altering the global expression of transporters genes.

The only gene significantly downregulated following rapamycin treatment was the isoform of system A transporter, SLC38A4. In the placenta, it is regulated as a paternally imprinted gene and is involved in the epigenetic control of nutrient supply and demand during development [52]. SLC38A4 knockout mice presented a lower placenta weight causing a severe intrauterine growth restriction [53], while SLC38A4 protein was significantly downregulated in early preterm human pregnancies complicated with IUGR or preeclampsia, without relevant alterations on gene expression levels [54]. Differently, a lack of change in both protein and mRNA expression was reported in human trophoblast cells [51] subjected to the same rapamycin treatment applied in our study. Indeed, the discrepancy between mRNA and protein levels appears to be a feature of this family of nutrient transporters [18,51], making comparisons of different results particularly difficult.

Previous studies in ruminants reported temporal changes in SLC expression during the early stage of placentation. However, placenta obtained from nutrient-restricted ewes showed that the impact of reduced maternal nutrition on SLC expression is subtler, as detectable only in pregnancy which later develops IUGR [55]. In our *in vitro* experiments, we found that SLC expression levels did not change in oTCs exposed to starved conditions, with SLC43A2 being the only exception. In cattle, it has been reported that the expression of SLC43A2 increased as conceptus development progresses [32] while in humans it is described as a unique transporter that may mediate the net efflux of amino acid from the placenta to the fetus [56]. The evidence that its mRNA expression positively correlates with fetal growth [32] and our data on SLC43A2 downregulation following starvation suggest that it could represent a particularly sensitive hotspot for nutrient availability among genes regulating the placental system L of amino acid transporters.

Contrary to what is generally claimed regarding mTOR suppression and LC3B activation, it is interesting to note that the expression of phospho-Ser-2448-mTOR in starved cells was not reduced compared to control, notwithstanding the depletion of serum and glutamine which is a well-known activator of mTOR [57]. In light of this, we speculate the existence of a compensation mechanism thanks to which the nutrients obtained through autophagic recycling lead to the reactivation of mTOR to limit extreme degradation by autophagy (Fig. 5). The existence of a feedback mechanism involving the general amino acid control (GAAC) pathway, mTOR and autophagy has been reported in previous works using rat kidney cells subjected to prolonged serum/glutamine starvation [58,59]. According to our findings, prolonged starvation restores mTOR activity in an autophagy-dependent manner, while rapamycin abolishes this rescue mechanism [58]. Moreover, Chen and colleagues [59] proposed that increasing the activity of amino acid transporters leads to an increase in intracellular amino acid uptake, which in turn causes the reactivation of mTOR and suppression of autophagy. However, further research is needed to better explore SLC activity looking at protein instead of mRNA expression, which has proved to be of little use in this type of investigation.

The present study provides evidence of multi-level trophoblast cell adaptation during the early stage of placentation in sheep. Nutrient deprivation appears to have a positive impact on placenta functionality thanks to a balanced regulation between mTOR and autophagy. This mechanism aims to ensure cell survival under prolonged starvation by redistributing nutrients recycled through autophagy, thus leading to mTOR reactivation, whereas in rapamycin-treated cells mTOR restoration is not allowed. Accordingly, we can assume that when mTOR activity was inhibited by rapamycin treatment trophoblast cells lost their capacity to mitigate autophagy proceeding and thus abolished their

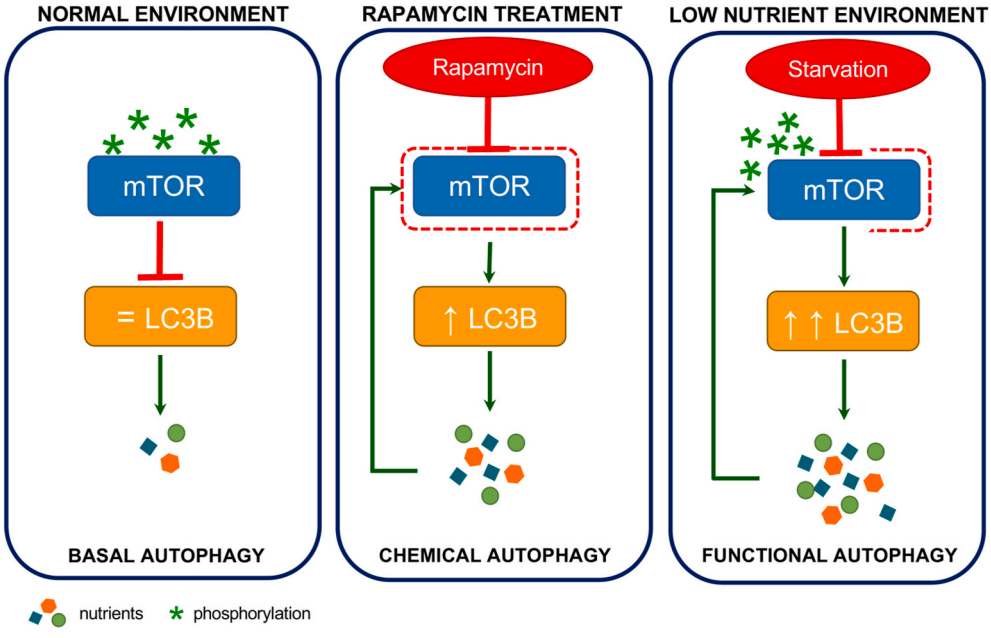


Fig. 5. Autophagy modulation in ovine trophoblast cells. Autophagy process modulation is controlled according to the different environments where the placenta is developing. In the presence of adequate nutrients availability, mTOR is activated and a baseline level of autophagy for normal recycling of nutrients is expected (=LC3B: basal autophagy). When trophoblast cells live in a rapamycin treatment system, mTOR is suppressed and an increase in autophagosome accumulation leads to a higher nutrient release in the cytoplasm. Despite the recycled nutrients, mTOR reactivation is not feasible due to the rapamycin-mediated complete inhibition. In that stressful environment, since mTOR does not fulfil its regulatory function, trophoblast cells may survive for a short time before dying (↑LC3B: chemical autophagy). On the other hand, a nutrient-deprived environment should potentially have an inhibitory effect on mTOR, leading to a much higher autophagosome synthesis. However, the greater release of new compounds seems to restore mTOR ability to provide new nutrient sources to support trophoblast cells functionality (↑↑LC3B: functional autophagy). In a low-nutrient context, mTOR acts as a balance between the nutrient sensor and autophagy regulator gate.

functional proprieties to preserve energy consumption (Fig. 5). Concluding, trophoblast may respond to a suboptimal *in vitro* environment by modulating nutrient recycling/transport and cellular functional activities, with potential implications for conceptus development and survival.

From a comparative perspective, sheep and human placentas are anatomically quite divergent based on the classification of Grosser [60]. In humans, the placenta truly invades the endometrium, whereas in sheep there is no massive erosion of the endometrium but a slight apposition of uterine and chorion layers [61]. However, trophoblast cells follow identical developmental steps during early pregnancy, including proliferation, differentiation, migration, and invasion [62]. Therefore, the sheep represents an appropriate model for human pregnancy due to similar physiological traits, such as body weight, litter size, and metabolic processes [63,64]. In particular, an increasing amount of research indicates that both species exhibit a similar mechanism for exchanging, transporting, and distributing nutrients and oxygen through placental tissue [63,65]. In this light, the compensatory response (Fig. 5) here proposed might be further investigated even in human gestational diseases sharing a common origin in defective placental development, such as intrauterine growth restriction [66,67] and preeclampsia [68,69].

Funding sources

This work was supported by a University of Turin, Italy Grant (TOSP_RILO_20_01) and a Grant for Internationalization (TOSP_GFI_22_01_F).

CRediT authorship contribution statement

Irene Viola: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paolo Accornero:** Methodology, Formal analysis. **Isabella Manenti:** Methodology, Formal analysis. **Silvia Miretti:** Formal analysis, Data curation. **Mario Baratta:** Writing – review & editing, Supervision. **Paola Toschi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2024.09.011.

References

[1] A.T. Grazul-Bilska, P.P. Borowicz, M.L. Johnson, M.A. Minten, J.J. Bilski, R. Wroblewski, D.A. Redmer, L.P. Reynolds, Placental development during early pregnancy in sheep: vascular growth and expression of angiogenic factors in the maternal placenta, Reprod 140 (2010) 165–174. https://doi:10.1530/REP-13-0663.

[2] K.G. Pringle, K.L. Kind, A.N. Sferruzzi-Perri, J.G. Thompson, C.T. Roberts, Beyond oxygen: complex regulation and activity of hypoxia-inducible factors in pregnancy, Hum. Reprod. Update 16 (2010) 415–431. https://doi:10.1093/humupd/dmp046.

[3] M. Rebsamen, E. Girardi, V. Sedlyarov, S. Scorzoni, K. Papakostas, M. Vollert, J. Konecka, B. Guertl, K. Klavins, T. Wiedmer, G. Superti-Furga, Gain-of-function genetic screens in human cells identify SLC transporters overcoming environmental nutrient restrictions, Life Sci. Alliance 16 (2022) e202201404, https://doi.org/10.26508/lsa.202201404.

[4] K.R. Parzych, D.J. Klionsky, An overview of autophagy: morphology, mechanism, and regulation, Antiox, Redox Signal 20 (2014) 460–473. https://doi:10.1089/ars.2013.5371.

[5] H.Y. Wen, S. Abbasi, R.E. Kellems, Y. Xia, mTOR: a placental growth signaling sensor, Placenta 26 (2005) S63–S69. https://doi:10.1016/j.placenta.2005.02.004.

[6] X. Wang, G. Wu, F.W. Bazer, mTOR: the master regulator of conceptus development in response to uterine histotroph during pregnancy in ungulates, in: K. Maiese (Ed.), Molecules to Medicine with mTOR, Academic Press, San Diego, 2016, pp. 23–35, https://doi.org/10.1016/B978-0-12-802733-2.00016-5.

[7] S. Roos, T.L. Powell, T. Jansson, Placental mTOR links maternal nutrient availability to fetal growth, Biochem. Soc. Trans. 37 (2009) 295–298. https://doi:10.1042/BST0370295.

[8] I. Viola, P. Toschi, I. Manenti, P. Accornero, M. Baratta, Modulatory role of mTOR in trophoblast adaptive response in the early stage of placentation in sheep, Reproduire 165 (2023) 313–324. https://doi:10.1530/REP-22-0356.

[9] G.Y. Liu, D.M. Sabatini, mTOR at the nexus of nutrition, growth, aging and disease, Nat. Rev. Mol. Cell Biol. 21 (2020) 183–203. https://doi:10.1038/s41580-019-0199-y.

[10] T. Jansson, I.L. Aye, D.C. Goberdhan, The emerging role of mTORC1 signaling in placental nutrient-sensing, Placenta 33 (2012) 23–29. https://doi:10.1016/j.placenta.2012.05.010.

[11] K.M. Halloran, E.C. Hoskins, C. Stenhouse, R.M. Moses, K.A. Dunlap, M. C. Satterfield, H. Seo, G.A. Johnson, G. Wu, F.W. Bazer, Pre-implantation exogenous progesterone and pregnancy in sheep. II. Effects on fetal-placental development and nutrient transporters in late pregnancy, J. Anim. Sci. Biotechnol. 12 (2021) 46. https://doi:10.1186/s40104-021-00567-1.

[12] G. Van Gronigen Case, K.M. Storey, L.E. Parmeley, L.C. Schulz, Effects of maternal nutrient restriction during the periconceptional period on placental development in the mouse, PLoS One 16 (2021) e0244971. https://doi:10.1371/journal.pone.0244971.

[13] J.D. Glazier, I. Cetin, G. Perugino, S. Ronzoni, A.M. Grey, D. Mahendran, A. M. Marconi, G. Pardi, C.P. Sibley, Association between the activity of the system A amino acid transporter in the microvillous plasma membrane of the human placenta and severity of fetal compromise in intrauterine growth restriction, Pediatr. Res. 42 (1997) 514–519. https://doi:10.1203/00006450-199710000-00016.

[14] T. Jansson, V. Scholtbach, T.L. Powell, Placental transport of leucine and lysine is reduced in intrauterine growth restriction, Pediatr. Res. 44 (1998) 532–537. https://doi:10.1203/00006450-199810000-00011.

[15] S. Roos, N. Jansson, I. Palmberg, K. Säljö, T.L. Powell, T. Jansson, Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth, Phys. J. 582 (2007) 449–459. https://doi:10.1113/jphysiol.2007.129676.

[16] F.J. Rosario, N. Jansson, Y. Kanai, P.D. Prasad, T.L. Powell, T. Jansson, Maternal protein restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and down-regulates placental amino acid transporters, Endocrinology 152 (2011) 1119–1129. https://doi:10.1210/en.2010-1153.

[17] S. Roos, O. Lagerlöf, M. Wennergren, T.L. Powell, T. Jansson, Regulation of aminoacid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling, Am. J. Physiol. Cell Physiol. 297 (2009) C723–C731. https://doi:10.1152/ajpcell.00191.2009.

[18] F.J. Rosario, Y. Kanai, T.L. Powell, T. Jansson, Mammalian target of rapamycin signalling modulates amino acid uptake by regulating transporter cell surface abundance in primary human trophoblast cells, Phys. J. 591 (2013) 609–625. https://doi:10.1113/jphysiol.2012.238014.

[19] H. Gao, G. Wu, T.E. Spencer, G.A. Johnson, F.W. Bazer, Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses, Biol. Reprod. 80 (2009) 1196–1208. https://doi:10.1095/biolreprod.108.075440.

[20] L. Belkacemi, D.M. Nelson, M. Desai, M.G. Ross, Maternal undernutrition influences placental-fetal development, Biol. Reprod. 83 (2010) 325–331. https://doi:10.1095/biolreprod.110.084517.

[21] X. Yin, R. Gao, Y. Geng, X. Chen, X. Liu, X. Mu, Y. Ding, Y. Wang, J. He, Autophagy regulates abnormal placentation induced by folate deficiency in mice, Mol. Hum. Reprod. 25 (2019) 305–319. https://doi:10.1093/molehr/gaz022.

[22] J.S. Gong, G.S. Kim, The role of autophagy in the placenta as a regulator of cell death, Clin. Exp. Reprod. Med. 41 (2014) 97–107. https://doi:10.5653/cerm.2014.41.3.97.

[23] K.A. Vonnahme, B.W. Hess, T.R. Hansen, R.J. McCormick, D.C. Rule, G.E. Moss, W. J. Murdoch, M.J. Nijland, D.C. Skinner, P.W. Nathanielsz, S.P. Ford, Maternal undernutrition from early to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep, Biol. Reprod. 69 (2003) 133–140. https://doi:10.1095/biolreprod.102.012120.

[24] A. Chu, S. Thamotharan, A. Ganguly, M. Wadehra, M. Pellegrini, S.U. Devaskar, Gestational food restriction decreases placental interleukin-10 expression and markers of autophagy and endoplasmic reticulum stress in murine intrauterine growth restriction, Nutr. Res. 36 (2016) 1055–1067. https://doi:10.1016/j.nutres.2016.08.001.

[25] P. Toschi, M. Czernik, F. Zacchini, A. Fidanza, P. Loi, G.E. Ptak, Evidence of placental autophagy during early pregnancy after transfer of in vitro produced (IVP) sheep embryos, PLoS One 11 (2016) e0157594. https://doi:10.1371/journal.pone.0157594.

[26] A. Nakashima, A. Aoki, T. Kusabiraki, T. Shima, O. Yoshino, S.B. Cheng, S. Sharma, S. Saito, Role of autophagy in oocytogenesis, embryogenesis, implantation, and

pathophysiology of pre-eclampsia, J. Obstet. Gynaecol. Res. 43 (2017) 633–643. https://doi:10.1111/jog.13292.
[27] K.D. Broad, E.B. Keverne, Placental protection of the fetal brain during short-term food deprivation, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 15237–15241. https://doi:10.1073/pnas.1106022108.
[28] S. Chakraborty, R. Bose, S. Islam, S. Das, R. Ain, Harnessing autophagic network is essential for trophoblast stem cell differentiation, Stem Cell. Dev. 29 (2020) 682–694. https://doi:10.1089/scd.2019.0296.
[29] M. Gauster, S. Maninger, M. Siwetz, A. Deutsch, A. El-Heliebi, D. Kolb-Lenz, U. Hiden, G. Desoye, F. Herse, A. Prokesch, Downregulation of p53 drives autophagy during human trophoblast differentiation, Cell. Mol. Life Sci. 75 (2018) 1839–1855. https://doi:10.1007/s00018-017-2695-6.
[30] P. Toschi, I. Viola, I. Manenti, S. Miretti, E. Macchi, E. Martignani, P. Accornero, M. Baratta, Ovine trophoblast cells: cell isolation and culturing from the placenta at the early stage of pregnancy, in: M. Baratta (Ed.), Epithelial Cell Culture, Methods in Molecular Biology, Humana, New York, 2024, pp. 123–133.
[31] G.E. Ptak, P. Toschi, A. Fidanza, M. Czernik, F. Zacchini, J.A. Modlinski, P. Loi, Autophagy and apoptosis: parent-of-origin genome-dependent mechanisms of cellular self-destruction, Open Biol 4 (2014) 140027. https://doi:10.1098/rsob.140027. PMID: 24898141.
[32] N. Forde, C.A. Simintiras, R. Sturmey, S. Mamo, A.K. Kelly, T.E. Spencer, F. W. Bazer, P. Lonergan, Amino acids in the uterine luminal fluid reflects the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle, PLoS One 9 (2014) e100010. https://doi:10.1371/journal.pone.0100010.
[33] A. Suarez-Arnedo, F. Torres Figueroa, C. Clavijo, P. Arbeláez, J.C. Cruz, C. Muñoz-Camargo, An image J plugin for the high throughput image analysis of in vitro scratch wound healing assays, PLoS One 15 (2020) e0232565. https://doi:10.1371/journal.pone.0232565.
[34] P.M. Taylor, Role of amino acid transporters in amino acid sensing, Am. J. Clin. Nutr. 99 (2014) 223S–230S. https://doi:10.3945/ajcn.113.070086.
[35] R. Hyde, P.M. Taylor, H.S. Hundal, K. Peyrollier, G.R. Christie, Amino acid transporters: roles in amino acid sensing and signaling in animal cells, Biochem. J. 373 (2003) 1–18. https://doi:10.3945/ajcn.113.070086.
[36] N. Mizushima, T. Yoshimori, How to interpret LC3 immunoblotting, Autophagy 3 (2007) 542–545. https://doi:10.4161/auto.4600.
[37] J.Y. Kim, R.C. Burghardt, G. Wu, G.A. Johnson, T.E. Spencer, F.W. Bazer, Select nutrients in the ovine uterine lumen. VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration, Biol. Reprod. 84 (2011) 62–69. https://doi:10.1095/biolreprod.110.085738.
[38] A. Nakashima, A. Furuta, K. Yamada, M. Yoshida-Kawaguchi, A. Yamaki-Ushijima, I. Yasuda, M. Ito, S. Yamashita, S. Tsuda, S. Yoneda, S. Cheng, S. Sharma, T. Shima, The role of autophagy in the female reproduction system: for beginners to experts in this field, Biology 12 (2023) 373. https://doi:10.3390/biology12030373.
[39] A. Aoki, A. Ushijima, Y. Ono, S. Tsuda, T. Shima, O. Yoshino, H. Sago, K. Matsumoto, S. Sharma, S. Saito, Endoplasmic reticulum stress disrupts lysosomal homeostasis and induces blockade of autophagic flux in human trophoblasts, Sci. Rep. 9 (2019) 11466. https://doi:10.1038/s41598-019-47607-5.
[40] M.R. Karim, T. Kanazawa, Y. Daigaku, S. Fujimura, G. Miotto, M. Kadowaki, Cytosolic LC3 ratio as a sensitive index of macroautophagy in isolated rat hepatocytes and H4-II-E cells, Autophagy 3 (2007) 553–560. https://doi:10.4161/auto.4615.
[41] M.I. Koukourakis, D. Kalamida, A. Giatromanolaki, C.E. Zois, E. Sivridis, S. Pouliliou, A. Mitrakas, K.C. Gatter, A.L. Harris, Autophagosome proteins LC3A, LC3B and LC3C have distinct subcellular distribution kinetics and expression in cancer cell lines, PLoS One 10 (2015) e0137675. https://doi:10.1371/journal.pone.0137675.
[42] R. Huang, Y. Xu, W. Wan, X. Shou, J. Qian, Z. You, B. Liu, C. Chang, T. Zhou, J. Lippincott-Schwartz, W. Liu, Deacetylation of nuclear LC3 drives autophagy initiation under starvation, Mol. Cell. 57 (2015) 456–466. https://doi:10.1016/j.molcel.2014.12.013.
[43] Z. Dou, C. Xu, G. Donahue, T. Shimi, J.A. Pan, J. Zhu, A. Ivanov, B.C. Capell, A. M. Drake, P.P. Shah, J.M. Catanzaro, M.D. Ricketts, T. Lamark, S.A. Adam, R. Marmorstein, W.X. Zong, T. Johansen, R.D. Goldman, P.D. Adams, S.L. Berger, Autophagy mediates degradation of nuclear lamina, Nature 527 (2015) 105–109. https://doi:10.1038/nature15548.
[44] M.S. Shim, A. Nettesheim, J. Hirt, P.B. Liton, The autophagic protein LC3 translocates to the nucleus and localizes in the nucleolus associated to NUFIP1 in response to cyclic mechanical stress, Autophagy 16 (2020) 1248–1261. https://doi:10.1080/15548627.2019.1662584.
[45] L.J. Kraft, P. Manral, J. Dowler, A.K. Kenworthy, Nuclear LC3 associates with slowly diffusing complexes that survey the nucleolus, Traffic 17 (2016) 369–399. https://doi:10.1111/tra.12372.
[46] S.Y. Oh, C.R. Roh, Autophagy in the placenta, Obstet. Gynecol. Sci. 60 (2017) 241–259. https://doi:10.5468/ogs.2017.60.3.241.
[47] J.A. Green, R.D. Geisert, G.A. Johnson, T.E. Spencer, Implantation and placentation in ruminants, in: R.D. Geisert, T.E. Spencer (Eds.), Placentation in Mammals. Advances in Anatomy, Embryology and Cell Biology, Springer Nature, Cham, 2021, pp. 129–154.
[48] U.M. Igwebuike, Trophoblast cells of ruminant placentas-A minireview, Anim. Reprod. Sci. 93 (2006) 185–198. https://doi:10.1016/j.anireprosci.2005.06.003.
[49] F.B.P. Wooding, The ruminant placental trophoblast binucleate cell: an evolutionary breakthrough, Biol. Reprod. 107 (2022) 705–716. https://doi:10.1016/j.anireprosci.2005.06.003.
[50] M. Hiyama, K.T. Kusakabe, A. Takeshita, S. Sugi, N. Kuniyoshi, H. Imai, K. Kano, Y. Kiso, Nutrient starvation affects the expression of LC3 family at the feto-maternal interface during murine placentation, J. Vet. Med. Sci. 77 (2015) 305–311. https://doi:10.1292/jvms.14-0490.
[51] S. Roos, Y. Kanai, P.D. Prasad, T.L. Powell, T. Jansson, Regulation of placental amino acid transporter activity by mammalian target of rapamycin, Am. J. Physiol. Cell Physiol. 296 (2009) C142–C150. https://doi:10.1152/ajpcell.00330.2008.
[52] M. Constancia, E. Angiolini, I. Sandovici, P. Smith, R. Smith, G. Kelsey, W. Dean, A. Ferguson-Smith, C.P. Sibley, W. Reik, A. Fowden, Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 19219–19224. https://doi:10.1073/pnas.0504468103.
[53] S. Matoba, S. Nakamuta, K. Miura, M. Hirose, H. Shiura, T. Kohda, N. Nakamuta, A. Ogura, Paternal knockout of Slc38a4/SNAT4 causes placental hypoplasia associated with intrauterine growth restriction in mice, Proc. Natl. Acad. Sci. U.S.A. 116 (2019) 21047–21053. https://doi:10.1073/pnas.1907884116.
[54] E. Kadife, A. Harper, N. De Alwis, K. Chien, N. Hannan, F.C. Brownfoot, SLC38A4 amino acid transporter expression is significantly lower in early preterm intrauterine growth restriction complicated placentas, Int. J. Mol. Sci. 24 (2023) 403. https://doi:10.3390/ijms24010403.
[55] K.A. Dunlap, J.D. Brown, A.B. Keith, M.C. Satterfield, Factors controlling nutrient availability to the developing fetus in ruminants, J. Anim. Sci. Biotechnol. 6 (2015) 16. https://doi:10.1186/s40104-015-0012-5.
[56] J.K. Cleal, J.D. Glazier, G. Ntani, S.R. Crozier, P.E. Day, N.C. Harvey, S. M. Robinson, C. Cooper, K.M. Godfrey, M.A. Hanson, R.M. Lewis, Facilitated transporters mediate net efflux of amino acids to the fetus across the basal membrane of the placental syncytiotrophoblast, Phys. J. 589 (2011) 987–997. https://doi:10.1113/jphysiol.2010.198549.
[57] J. Kim, G. Song, G. Wu, H. Gao, G.A. Johnson, F.W. Bazer, Arginine, leucine, and glutamine stimulate proliferation of porcine trophectoderm cells through the MTOR-RPS6K-RPS6-EIF4EBP1 signal transduction pathway, Biol. Reprod. 88 (2013) 113. https://doi:10.1095/biolreprod.112.105080.
[58] L. Yu, C.K. McPhee, L. Zheng, G.A. Mardones, Y. Rong, J. Peng, N. Mi, Y. Zhao, Z. Liu, F. Wan, D.W. Hailey, V. Oorschot, J. Klumperman, E.H. Baehrecke, M. J. Lenardo, Termination of autophagy and reformation of lysosomes regulated by mTOR, Nature 465 (2010) 942–946. https://doi:10.1038/nature09076.
[59] R. Chen, Y. Zou, D. Mao, D. Sun, G. Gao, J. Shi, X. Liu, C. Zhu, M. Yang, W. Ye, Q. Hao, R. Li, L. Yu, The general amino acid control pathway regulates mTOR and autophagy during serum/glutamine starvation, J. Cell Biol. 206 (2014) 173–182. https://doi:10.1083/jcb.201403009.
[60] O. Grosser, in: W. Braumüller (Ed.), Vergleichende anatomie und entwicklungsgeschichte der eihäute und der placenta: mit besonderer berücksichtigung des menschen, 1909. Vienna and Leipzig.
[61] J.H.J. Stegeman, Placental development in the sheep and its relation to fetal development. A qualitative and quantitative anatomic and histologic study, Bijdr. Dierkd. 44 (1974) 3–72.
[62] F.B.P. Wooding, A.P.F. Flint, Placentation, in: G.E. Lamming GE (Ed.), Marshall's Physiology of Reproduction, Springer, Dordrecht, 1994, pp. 233–460. https://doi:10.1007/978-94-011-1286-4_42.
[63] J.S. Barry Js, R.V. Anthony, The pregnant sheep as a model for human pregnancy, Theriogenology 69 (2008) 55–67. https://doi:10.1016/j.theriogenology.2007.09.021.
[64] J.L. Morrison, M.J. Berry, K.J. Botting, J.R.T. Darby, M.G. Frasch, K.L. Gatford, D. A. Giussani, C.L. Gray, R. Harding, E.A. Herrera, M.W. Kemp, M.C. Lock, I. C. McMillen, T.J. Moss, G.C. Musk, M.H. Oliver, T.R.H. Regnault, C.T. Roberts, J. Y. Soo, R.L. Tellam, Improving pregnancy outcomes in humans through studies in sheep, Am. J. Physiol. Regul. Integr. Comp. Physiol. 315 (2018) 1123–1153. https://doi:10.1152/ajpregu.00391.2017.
[65] I. Ribitsch, S. Chang-Rodriguez, M. Egerbacher, S. Gabner, S. Gueltekin, J. Huber, T. Schuster, F. Jenner, Sheep placenta cotyledons: a noninvasive source of ovine mesenchymal stem cells, tissue engineering - Part C, Methods 23 (2017) 298–310. https://doi:10.1089/ten.tec.2017.0067.
[66] S. Zhang, T.R.H. Regnault, P.L. Barker, K.J. Botting, I.C. McMillen, C.M. McMillan, C.T. Roberts, J.L. Morrison, Placental adaptations in growth restriction, Nutrients 7 (2015) 360–389. https://doi:10.3390/nu7010360.
[67] T.H. Hung, S.F. Chen, L.M. Lo, M.J. Li, Y.L. Yeh, T.T. Hsieh, Increased autophagy in placentas of intrauterine growth-restricted pregnancies, PLoS One 7 (2012) e40957. https://doi:10.1371/journal.pone.0040957.
[68] B. Huppertz, Placental origins of preeclampsia: challenging the current hypothesis, Hypertension 51 (2008) 970–975. https://doi:10.1161/HYPERTENSIONAHA.107.107607.
[69] A. Nakashima, M. Yamanaka-Tatematsu, N. Fujita, K. Koizumi, T. Shima, T. Yoshida, T. Nikaido, A. Okamoto, T. Yoshimori, S. Saito, Impaired autophagy by soluble endoglin, under physiological hypoxia in early pregnant period, is involved in poor placentation in preeclampsia, Autophagy 9 (2013) 303–316. https://doi:10.4161/auto.22927.