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(Article begins on next page)

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RESEARCH

Modulatory role of mTOR in trophoblast adaptive response in the early stage of placentation in sheep

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Abstract

In brief: Fibroblast growth factor-2 (FGF2) is essential for early placenta development in sheep. This study shows that the mechanistic target of rapamycin is the key modulator of trophoblast adaptive response under FGF2 modulation.

Abstract: During the early stage of placentation in sheep, normal conceptus development is affected by trophoblast cell functionality, whose dysregulation results in early pregnancy loss. Trophoblast metabolism is supported mainly by histotrophic factors, including fibroblast growth factor-2 (FGF2), which are involved in cell differentiation and function through the modulation of specific cellular mechanisms. The mechanistic target of rapamycin (mTOR) is known as a cellular 'nutrient sensor', but its downstream regulation remains poorly understood. The hypothesis was that during trophoblast development, the FGF2 effect is mediated by mTOR signalling pathway modulation. Primary trophoblast cells from 21-day-old sheep placenta were characterised and subjected to FGF2 and rapamycin treatment to study the effects on cell functionality and gene and protein expression profiles. The model showed mainly mononuclear cells with epithelial cell-like growth and placental morphological properties, expressing typical trophoblast markers. FGF2 promoted cell proliferation and migration under normal culture conditions, whereas mTOR inhibition reversed this effect. When the mTOR signalling pathway was activated, FGF2 failed to influence invasion activity. mTOR inhibition significantly reduced cell motility, but FGF2 supplementation restored motility even when mTOR was inhibited. Interestingly, mTOR inhibition influenced endocrine trophoblast marker regulation. Although FGF2 supplementation did not affect ovine placenta lactogen expression, as observed in the control, interferon-tau was drastically reduced. This study provides new insights into the mechanism underlying mTOR inhibitory effects on trophoblast cell functionality. In addition, as mTOR is involved in the expression of hormonal trophoblast markers, it may play a crucial role in early placenta growth and fetal–maternal crosstalk.

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Introduction

The placenta primarily comprises trophoblast cells that develop from the embryonic trophectoderm during the blastocyst stage (Igwebuike 2006). In sheep, after conceptus reaches the maximum elongation (from day 16) (Spencer *et al.* 2004), the trophectoderm completely adheres to the maternal endometrium on day 21 and differentiates to establish pregnancy (Johnson *et al.* 2018). From this moment, the placenta plays a pivotal role in balancing sufficient oxygen, nutrient and waste exchange to safeguard normal embryonic growth and survival.

In the early stage of placentation, as a result of inadequate vascularisation (Grazul-Bilska *et al.* 2010, Devi *et al.* 2020) and the low uterus–conceptus interaction, trophoblast cells adapt to overcome a nutrient-poor and hypoxic environment (Pringle *et al.* 2010). However, trophoblast cell metabolism is entirely supported by the endometrial histotroph, which is a source of multiple components, including hormones, sugars, amino acids, enzymes and growth factors (Bazer *et al.* 2011). Several uterine-derived growth factors are required for ovine conceptus implantation (Bazer *et al.* 2015), one among which is fibroblast growth factor-2 (FGF2) (Ocòn-Grove *et al.* 2008, Yang *et al.* 2011*a*, Chiumia *et al.* 2020). FGF2 is involved in trophoblast functionality in supporting normal placental development by stimulating mitogen activity (Xie *et al.* 2017, Bonometti *et al.* 2019), differentiation (Pfarrer *et al.* 2006) and interferon-tau (IFNT) secretion (Yang *et al.* 2011*b*).

Different intracellular signalling pathways, such as PI3/Akt (Wang *et al.* 2021), MAPK (Lim *et al.* 2018) and ERK1/2 (Bonometti *et al.* 2019), mediate the effects of FGF2 on trophoblast cells. Notably, FGF2 is also involved in the mechanistic target of rapamycin (mTOR) activation – a paramount 'nutrient-sensing'

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pathway in conceptus development (Bazer *et al.* 2011, Lin *et al.* 2011, Wang *et al.* 2016). mTOR is essential in controlling trophoblast cell growth and migration in response to nutrient and growth factors during the peri-implantation period (Wen *et al.* 2005, Gupta & Jansson 2019). Therefore, low nutrient availability severely impairs mTOR trophoblast downstream regulation by affecting placental homeostasis (Toschi & Baratta 2021). Dysfunctions of placenta development due to changes in the proliferation, differentiation and migration of trophoblasts can result in gestational diseases, including early pregnancy loss and intrauterine growth restriction (Belkacemi *et al.* 2010, Chu *et al.* 2016).

As intrauterine environment changes directly impact placenta development by affecting livestock fertility, we investigated whether FGF2 affects trophoblast functionality through mTOR signalling pathway modulation. An *in vitro* culture system using an early sheep placenta (day 21) was established. This cellular model can be used to explore how the trophoblast regulates its adaptive response in terms of trophoblast marker expression in suboptimal environments, such as FGF2 deficiency and mTOR inhibition, to mimic adverse situations occurring in the early stages of pregnancy.

Materials and methods

Materials

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co. For cell isolation and culture, the following chemicals were used: Dulbecco's 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); FGF2, rh-FGF-basic (11343625; ImmunoTools GmbH, Gladiolenwag, Friesoythe, Germany); trypsin-EDTA (T2601); collagenaseIV (C5138); bovine serum albumin (BSA; A3733); DNaseI (260913), rapamycin (Santa Cruz, sc-3504A); and dimethyl sulfoxide (DMSO; D2650).

For western blotting and immunocytochemistry analyses, the following antibodies were used: mouse anti-alpha-tubulin (1:10,000, T5168); mouse anti-STAT3 (1:10,000, Cell Signalling, 9131S); rabbit anti-mTOR (1:1000, Cell Signalling, 2983); rabbit anti-pmTOR (1:1000, Cell Signalling, 5536) and rabbit anti-pAkt (1:1000, Cell Signalling, 92715); mouse anti-p70 (1:1000, Santa Cruz Biotechnology, sc-8418); mouse anti-cytokeratin7 (1:100, Santa Cruz Biotechnology, sc-23876); mouse anti-vimentin (1:300, Santa Cruz Biotechnology, sc-32322); 4′,6-diamidino-2-phenylindole (DAPI; Thermo Scientific); Alexa-fluor-594 goat-anti-mouse (1:500, Invitrogen, A11032); Alexa-fluor-488 goat-anti-mouse (1:500, Invitrogen, A32723); and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:15,000, Immunopure Goat anti-rabbit and anti-mouse IgG, Thermo Fisher Scientific). 5-Bromo-2′-deoxyuridine (BrdU) (19160), anti-BrdU (1:100, B2531), growth factor-reduced Matrigel Matrix (BD Biosciences, San Jose, CA, USA; 354230) and crystal violet (C3886) were used for cell functionality tests.

The experiments were conducted using two cellular models: the oTr cell line (day 15 sheep placenta, kindly provided by Dr Fuller Bazer) and ovine primary trophoblast cells (oTCs) directly obtained from early pregnant uterus. The experimental design is shown in Fig. 1.

Placenta collection and ovine trophoblast cell isolation

Uteri from pregnant sheep were collected at the slaughterhouse and immediately placed in a sterile pack with sterile phosphate-buffered saline (PBS) pH 7.2 solution for transport in a thermal container. The pregnancy stage was estimated by measuring the embryo crown–rump length, and 21- to 23-day-old placentae ($n=3$) were used (Ptak *et al.* 2013). The uteri were quickly

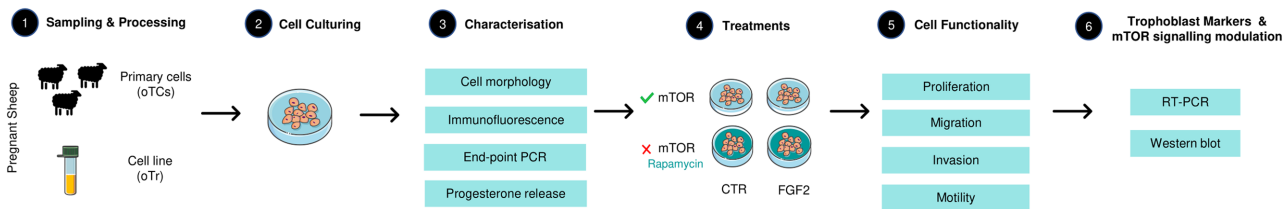

Figure 1 Experimental design. The main steps of the study are shown in the experimental design flow. *Sampling and Processing* (step 1): primary trophoblast cells were collected at the slaughterhouse from sheep ($n=3$) on days 21–23 of pregnancy and processed by mechanic and enzymatic digestion method for cell isolation (oTCs); an established cell line from sheep early trophoblast was also used in the following steps. *Cell culturing* (step 2): cells were cultured in a supplemented growth medium at 37°C in an atmosphere of 5% CO_2; *Characterisation* (step 3): cells were characterised in terms of cell morphology and specific trophoblast marker detection. First, oTCs and oTr phenotype was observed by cell microscopy for 7 days to follow the typical trophoblast cell differentiation in each culture model. Then, trophoblast marker detection was performed by immunofluorescence cell staining, endpoint PCR and immunoenzymatic methods for progesterone release in the culture medium. *Treatments* (step 4): cells were subjected to different 24 h treatments in order to study trophoblast behaviour in mTOR-activated/inhibited system (by adding rapamycin, a selective inhibitor of mTOR), with or without FGF2 supplementation. *Cell functionality* (step 5): the effect of previous treatments on oTCs and oTr was explored by BrdU incorporation assay for cell proliferation, while Transwell (with or without Matrigel coated) and wound-healing assay were performed for cell migration, invasion and motility. *Trophoblast markers and mTOR signalling modulation* (step 6): in order to explore the effect of FGF2 on mTOR target, the expression of structural and hormonal trophoblast markers was studied thanks to RT-PCR; western blot analysis was assessed for mTOR axis modulation.

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cleaned with 70% ethanol for 1 s and transferred to a sterile Petri dish once they arrived in the laboratory within 1 h of being collected. Uterine horn dissection was performed using sterile surgical instruments, and the placenta was manually separated from the maternal endometrium and repeatedly washed in sterile PBS pH 7.2 solution, supplemented with 10 UI/L penicillin and 10 µg/mL streptomycin.

Cell isolation was performed by mechanical and enzymatic disaggregation. Using sharp fine-point scissors and blunt forceps, the placenta was separated from the embryo and placed in a sterile Petri dish. The tissue was minced finely with a blade to obtain viscous and bloody tissue and transferred into a falcon tube with 50 mL digestion solution, comprising 25 mg trypsin–EDTA, 25 mg collagenaseIV, 50 mg BSA and 0.2 mg/mL DNaseI in 50 mL of DMEM for 90 min at 37°C in a shaking incubator adjusted to 100 moves/min. The supernatant was filtered through a 100-µm mesh-size cell strainer to remove undigested tissue fragments, and enzymatic digestion was stopped by adding 10% FBS. The filtrate was centrifuged at 480 ***g*** for 10 min at room temperature (RT). The pellet was washed with 5 mL of sterile distilled water for 10 s to remove blood cell contamination, after which 5 mL of PBS 2× were immediately added to equilibrate the osmolarity solution. The solution was centrifuged again at 1480 ***g*** for 10 min.

Cells were placed in a Petri dish in trophoblast growth medium defined as control (CTR), comprising 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% CO_2, and the culture medium was replaced every 48 h until 80% confluence.

Treatments

For all following experiments, cells were starved for 24 h with DMEM lacking all other supplements and then subjected to different treatments (CTR: trophoblast growth medium; FGF2: trophoblast growth medium + 50 ng/mL FGF2; rapa-CTR: trophoblast growth medium + 100 nM rapamycin; rapa-FGF2: trophoblast growth medium + 50 ng/mL FGF2 and 100 nM rapamycin). Cells were also treated with DMSO only (a solvent of rapamycin) as a vehicle control. All experiments were conducted using oTC primary cells between 2 and 8 passages and oTr cell between 14 and 20 passages. All experiments, unless otherwise indicated, were performed at least three times starting from different cell batch for both oTCs and oTr.

Proliferation assay

Cell proliferation was performed using a BrdU incorporation assay on oTCs and oTr cultured for 12, 24 and 48 h under CTR and FGF2 treatment conditions; 25,000 cells were seeded on a four-well chamber slide in duplicate for each treatment (Nunc Lab-Tek II Chamber Slide system) at 60% confluence and starved for 24 h. After that, cells were incubated with 10 µM BrdU, fixed in 4% formaldehyde for 15 min and permeabilised at RT with 0.1% Triton X-100 for 15 min. Then, the cells were treated with 2 M HCl at RT for 30 min; blocking was performed using 1% BSA at RT for 1 h, and anti-BrdU was added and incubated overnight at 4°C. Subsequently, the cells were incubated at RT for 1 h with the secondary antibody Alexa-fluor-488 goat-anti-mouse and then counterstained with DAPI for 10 min at RT. Between every step, the cells were washed three times with PBS at RT for 2 min. The number of proliferative cells was calculated for each group by calculating the ratio between the number of BrdU-positive cells and the total number of nuclei. At least 1000 nuclei were analysed for each treatment by randomly selecting 20 fields. Images were acquired with a Leica AF6000 LX (Leica Microsystems, Wetzlar, Germany) fluorescent microscope, equipped with a Leica DFC350FX digital camera controlled by LAS AF software (Leica Microsystems).

Migration and invasion assay

oTCs and oTr migration was conducted using 24-well Transwell plates; 50,000 cells in 100 µL of serum- and insulin-free DMEM were seeded into the upper chamber of an 8-µm Transwell chamber (Costar, 3422, Corning, NY, USA), to which 600 µL of growth medium were added, according to the treatment, into the lower chamber. After 12 h of culture, the non-migrated cells on the upper side of the inserts were removed washing twice with PBS and using a sterile-cotton swab. To evaluate migration onto the lower surface, the inserts were fixed in 4% formaldehyde for 15 min. The dried chamber was stained with DAPI for 10 min at RT and washed with PBS, and the cells were then stained with 0.5% crystal violet for 20 min. Cells were counted in 25 random non-overlapping fields acquired at a magnification of 10×, and the whole pictures of the migration surface were obtained by using a Leica AF6000 LX (Leica Microsystems) fluorescent microscope. An invasion assay was performed following the same procedure, but the Transwell was coated with Matrigel (Pijuan *et al.* 2019).

Wound-healing assay

About 50,000 oTCs were seeded in 24-well plates until 80% confluent cell monolayer starved for 12 h. Then, a straight line was drawn with a sterile p-200 pipette tip in the centre of the dish. Detached cells were removed by washing twice with PBS, and a medium was added following the experimental conditions. Images were captured every 6 h with a Leica AF6000 LX and analysed with ImageJ Fiji 1.53s (National Institutes of Health, USA; https://imagej.nih.gov/ij/), following the standardised scratch wound-healing evaluation (Suarez-Arnedo *et al.* 2020). Each experiment was repeated in triplicate twice.

Immunocytochemistry

oTCs were cultured on four-well chamber slides (Nunc Lab-Tek II Chamber Slide system), using oTr and sheep embryonic fibroblast (SEF) as positive and negative controls, respectively. Cells were fixed with a suitable volume of 4% paraformaldehyde at RT for 10 min, followed by washing twice with Tris-buffered saline (TBS) for 3 min. Non-specific antigen sites were blocked with 10% goat serum at RT for 1

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h. Subsequently, fixed cells were incubated at RT for 1 h with the following antibodies: cytokeratin-7 (CK7) and vimentin (Vim). After extensive washing with TBS, cells were incubated at RT for 1 h in the dark with the secondary antibody Alexa-fluor-594 goat-anti-mouse. Simultaneously, the negative controls were processed similarly, except for the inclusion of primary antibodies. The slides were again washed with TBS, and DAPI was then added for 10 min at RT for nuclei staining. The pictures were acquired with a Leica SP8 confocal fluorescent microscope (Leica Microsystems).

Gene expression analyses

For RNA extraction, oTCs were seeded in six-well plates (3×10^5 cells/well) for both control and treatments. Total RNA was extracted by using a Maxwell RSC simplyRNA tissue kit (Promega) following the manufacturer's instructions. 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 Thermo Cycler, USA). For the quantitative polymerase chain reaction (qPCR), 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) following the manufacturer's protocol. Obtained cDNA and no-RT samples were used for gene expression analysis using specific 5′–3′ primer pairs designed by Primer3 (https://primer3.ut.ee) software (CK7; pregnancy-associated glycoprotein (PAG); FGF2) or selected from previously published papers (epithelial–cadherin (CDH1; Seo *et al*. 2019); ovine placental lactogen (oPL; Lacroix *et al*. 2002); IFNT (Sakurai *et al*. 2010); fibroblast growth factor receptor-2 (FGFR2; McCoski *et al*. 2018)) to anneal at 58°C/60°C with an amplification efficiency (E) range between 2.1 and 1.9 (Supplementary Table 1). Each run was performed in triplicate under conditions of 40 cycles of 94°C for 45 s, 58/60°C for 45 s and 72°C for 1 min. The relative expression of each gene analysed was calculated using the comparative threshold cycle method with ribosomal protein S9 (RPLS9; McCoski *et al*. 2018) and ribosomal protein L32 (RPL32) as housekeeping genes. To avoid false-positive signals, dissociation curve analyses and negative controls (no-sample) were performed in each run.

Endpoint PCR was run following the kit's procedure (Qiagen Multiplex PCR Plus Kit). The amplicons were separated by agarose (3%) gel electrophoresis at the end of a 60-min run. The gel was placed on an ultraviolet illuminator (Bio-Rad GelDoc XR Molecular Imager) for visualisation and imaging.

Western blotting

Protein extraction was performed on 90% confluent oTCs on six-well plates under the indicated experimental conditions. Cells were washed with ice-cold PBS and then lysed for 10 min on ice in 200 µL lysis solution (Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol and 1% Triton X-100), Protease Inhibitor Cocktail (1:100), 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Samples were scraped, collected and centrifuged at 4°C for 15 min at 15,000 × ***g***. Supernatants were quantified with DC Protein Assays (Bio-Rad Laboratories) following the protocol's instructions. For western blotting, samples (15 µg of total protein) were resolved on 7.5% polyacrylamide gels and transferred to 0.2-µm nitrocellulose blotting membranes (Amersham Protran Premium). Membranes were blocked at RT for 1 h 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 following primary antibodies: alpha-tubulin, STAT3, pAkt mTOR, pmTOR and p70. Membranes were washed in TBS–Tween and incubated at RT for 1 h with 1:15,000 diluted HRP-conjugated secondary antibodies. Membranes were washed in TBS–Tween and incubated for 5 min at RT with Clarity Western ECL Substrate (Bio-Rad Laboratories). The proteins were visualised by exposing the membranes to an autoradiographic CL-XPosure Film (Thermo Fisher Scientific). Western blotting results were acquired with an EPSON Perfection V39 scanner.

Progesterone extraction and detection assays

Progesterone extraction was performed following the DetectX® Steroid Liquid Sample Extraction Protocol provided by Arbor Assays, with slight modifications. Briefly, the culture medium of oTCs and oTr was collected and centrifuged for 15 min at 13,520 ***g*** to discard cellular debris. The suspension was stored at –80°C until extraction. In addition, 400 µL of suspension was processed with 2 mL of diethyl ether in a glass tube, vortexed for 1 min, left at RT for 5 min and stored for 1 h at −80°C. The ether was poured into a new tube. The procedure was repeated twice. Then, the ether was transferred to a glass vial for evaporation by ultracentrifugation in a speedvac for 3 h (Thermo Savant, East Lyme, CT, USA; SC110A-115 SpeedVac Plus Concentrator), closed with parafilm and stored at –20°C until processing. To determine the progesterone concentration, an enzyme-linked immunosorbent assay kit (Progesterone ELISA, DRG Diagnostics GmbH, Germany) was used, and samples were resuspended in 250 µL of PBS following the protocol. All analyses were repeated twice on three different endpoint analyses. The progesterone concentration was expressed as pg/100,000 cells.

Statistical analysis

For each assay, data were obtained from at least three different replicates. One-way analysis of variance was performed to analyse cell migration and invasion and gene expression data. If the effect treatments were significant and/or sample groups were less than three, the nonparametric Mann–Whitney *U*-test was assessed. Data are reported as the mean ± S.D. This analysis was used for the wound-healing assays, but data are shown as mean ± S.E.M. Cell proliferation was analysed using Fisher's exact test. Statistical differences were considered significant when P was <0.05 in all statistical analyses.

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Results

Primary sheep trophoblast cell characterisation

The morphological characteristics and expression of some typical trophoblast markers were evaluated in oTCs. oTCs grow as epithelial like monolayers, showing mononuclear cells with epithelial cell-like growth and placental morphological properties, such as binucleate cells and multinucleated syncytium-plaque formation. Briefly, mononuclear oTCs formed 'circular clusters' within 48 h from seeding, and then binucleate and trophoblast giant cells (tri- or multinucleate cells) were observed in the surrounding area. Similar morphological characteristics were also observed for the oTr cell line (Fig. 2). oTCs reached senescence within 10–12 passages.

Trophoblast marker expression was performed on oTCs in comparison with the oTr cell line and SEF, which were used as positive and negative controls, respectively (Fig. 3). oTCs and oTr showed the expression of specific trophoblast markers, such as CK7, whereas SEF was negative. SEF and oTr showed vimentin-positive staining, but the oTCs did not. Moreover, progesterone release was detected for oTCs and oTr in the culture medium (Fig. 3). PCR analyses on 21-day oTCs displayed the expression of trophoblastic structural (CK7, CDH1) and functional (IFN-τ, oPL and PAG11) markers (Fig. 3).

Cell proliferation

FGF2 modulated oTC and oTr proliferation (Fig. 4). In both models, the proliferation rate increased at 24 h in untreated cell cultures ($P < 0.0001$). An increase ($P < 0.05$) in mitogenic response by 50 ng/mL FGF2 supplementation was observed from 12 h in oTCs. FGF2 treatment enhanced the mitotic index in both oTC and oTr cells, starting at 24 h ($p < 0.0001$), compared with untreated controls.

Cell migration and invasion assays

To mimic a stressful environmental status, the cells were subjected to the aforementioned experimental conditions. Migration assays showed similar results in both oTr and oTC models (Fig. 5A). In the absence of rapamycin, FGF2 supplementation induced a higher migration ability than that in the control ($P < 0.05$ in oTr, $P < 0.001$ in oTCs). When the mTOR signalling pathway was inhibited by rapamycin, the FGF2 effect was not observed. However, differences were observed when we compared the migration rate in cultured conditions with and without mTOR inhibition ($P < 0.001$), except for trophoblast growth medium (CTR) modulation in oTCs.

The ability of trophoblast cells to degrade the extracellular matrix was evaluated using an invasion assay (Fig. 5B). No differences were observed for

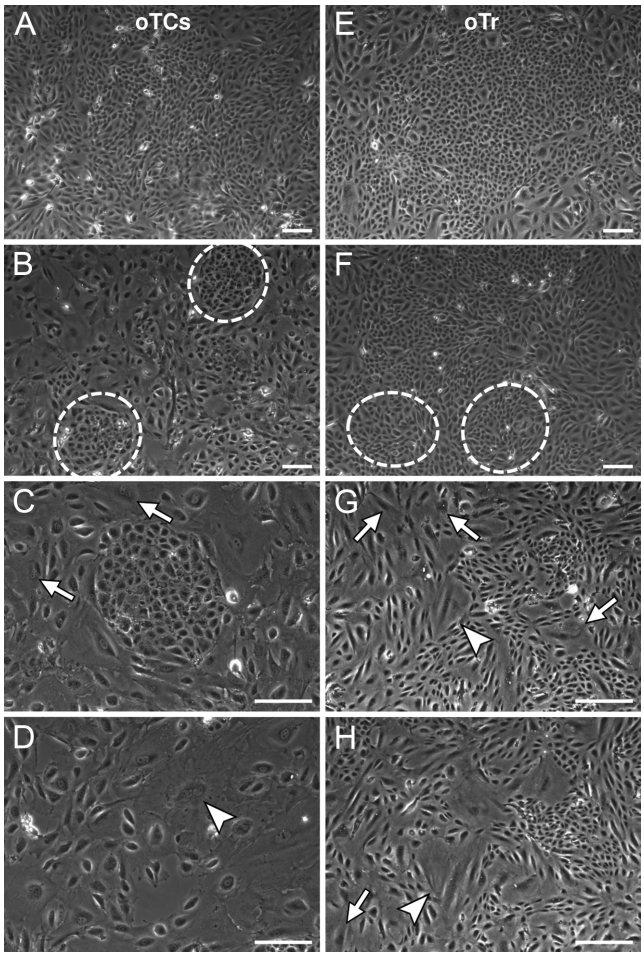

Figure 2 oTC primary cells and oTr cell line morphology. Twenty-one to twenty-three-day-old primary trophoblast cells were isolated from sheep uteri collected at the slaughterhouse. oTCs (ovine primary trophoblast cells) and oTr (ovine trophoblast cell line) were cultured with trophoblast growth medium and observed for 7 days. After seeding, mononuclear trophoblast pavement was observed on day 2 in both cell systems (A, E). Then, on day 4, mononuclear trophoblast cells started to form 'cobblestone-clusters'[1] (white dotted circles indicate initial cluster formation) (B, F). In peripheral areas of each cluster (C, D, G, H), trophoblast syncytial plaques were characterised by binuclear (BNC, arrows) and trophoblast giant cells (TCG, arrowheads). A, B, E, F: 5× magnification; C, D, G, H: 10× magnification. Scale bar at both magnifications: 100 µm.

oTC invasiveness when cells were cultured without rapamycin supplementation; when it was added, an increase was shown in the control compared to FGF2-supplemented medium ($P < 0.01$). As observed in the migration assay, even in this test, mTOR inhibition decreased oTC invasive ability ($P < 0.001$). In the oTr cell line, FGF2 did not affect invasion activity under normal culture conditions. When the mTOR signalling cascade was inhibited, FGF2 seemed to restore cell invasion ($P < 0.001$). Overall, oTr invasion was decreased in the mTOR-inhibited medium ($P < 0.001$), as also obtained in oTCs.

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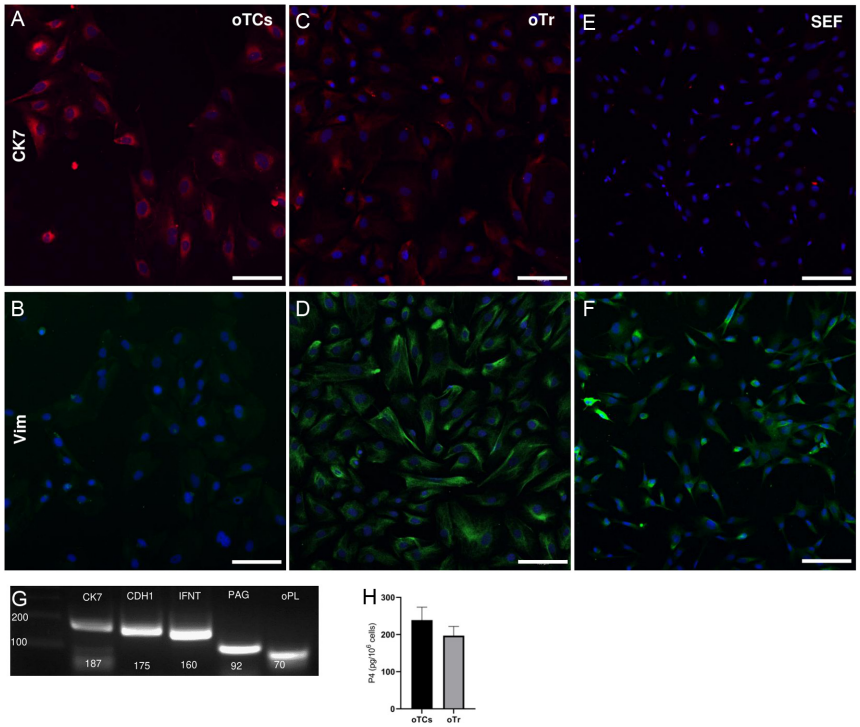

Figure 3 oTC characterisation. Immunofluorescence staining was performed on oTCs (ovine primary trophoblast cells, A, B), oTr (ovine trophoblast cell line, C, D) as a positive control and SEF (sheep embryonic fibroblast, E, F) as a negative control. Cells were cultured in growth medium until 70% confluence and fixed with 4% paraformaldehyde. Then cells were treated with 10% goat serum and incubated with cytokeratin7 (CK7, red staining) and vimentin (Vim, green staining) antibodies tested at 1:100 and 1:300 dilution, respectively. oTCs and oTr showed CK7 positively, whereas SEF was negative. Pictures were captured using the same confocal microscope parameters (20× magnification; scale bar: 100 µm). Conventional PCR (G) was conducted three times on oTCs in order to show structural and hormonal trophoblast marker genes. oTCs display a cytokeratin7 (*CK7*), e-cadherin (*CDH1*), interferon-τ (*IFNT*), protein related to pregnancy (*PAG*) and ovine placental lactogen (*oPL*) expression. Moreover, progesterone (P4) release was detected in oTCs and oTr (H). The culture medium was centrifuged to discard cellular debris and processed with diethyl ether at 80°C. From each medium sample progesterone was extracted for evaporation by ultracentrifugation. Then, progesterone concentration was evaluated by enzyme-linked immunosorbent assay kit and expressed as pg/100,000 cells. All analyses were repeated three different times. Both cellular models showed progesterone release in their growth medium.

Wound-healing assay

To test two-dimensional cell motility, oTC functionality was also evaluated using a scratch wound-healing assay. Track gap closure was followed over time from 0 to 36 h (Fig. 6). mTOR decreased cell motility starting from 18 h ($P < 0.01$). Furthermore, FGF2 supplementation promoted a cell-free gap filling within 24 h compared to the control ($P < 0.05$) and rapamycin treatment ($P < 0.01$). However, despite mTOR inhibition, FGF2 increased cell migration from 12 h, reaching complete coverage at 36 h.

Gene expression

Expression profiles for different genes involved in trophoblast cell functions (*CDH1*, *IFNT* and *oPL*) and FGF2 signalling (*FGF2* and *FGFR2*) were performed in above-mentioned treatments (Fig. 7A and B). qPCR analyses showed that FGF2 supplementation increased *CDH1* ($P < 0.0002$) and *oPL* ($P < 0.003$) expression, whereas *IFNT*, *FGF2* and *FGFR2* expression was similar between the control and treated groups. Moreover, rapamycin-treated cells showed a decrease in *IFNT*

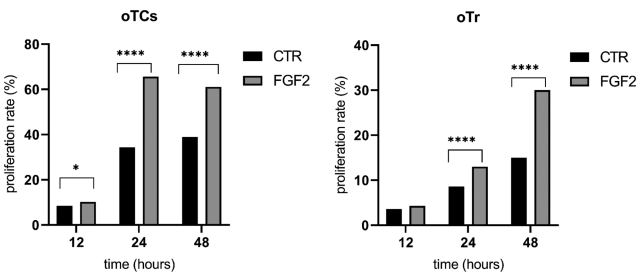

Figure 4 FGF2 effect on proliferation of oTCs and oTr. Cells were seeded in four-well chamber slides, starved at 60% of confluence for 24 h and then cultured with 50 ng/mL FGF2. Cell number was determined after 12, 24 and 48 h of incubation using BrdU incorporation assay. The total amount of proliferative cells was calculated by the ratio between the number of BrdU-positive and total number of nuclei. Thousand nuclei were considered by randomly selecting non-overlapping fields. Results are reported as the percentage of BrdU-positive cells of the total cell number counted. Proliferate assay was repeated three different times and data were analysed by Fisher's exact test (*$P < 0.05$; ****$P < 0.0001$). The graphs reveal that FGF2 increased cellular mitogen activity of both oTCs and oTr starting from 24 h.

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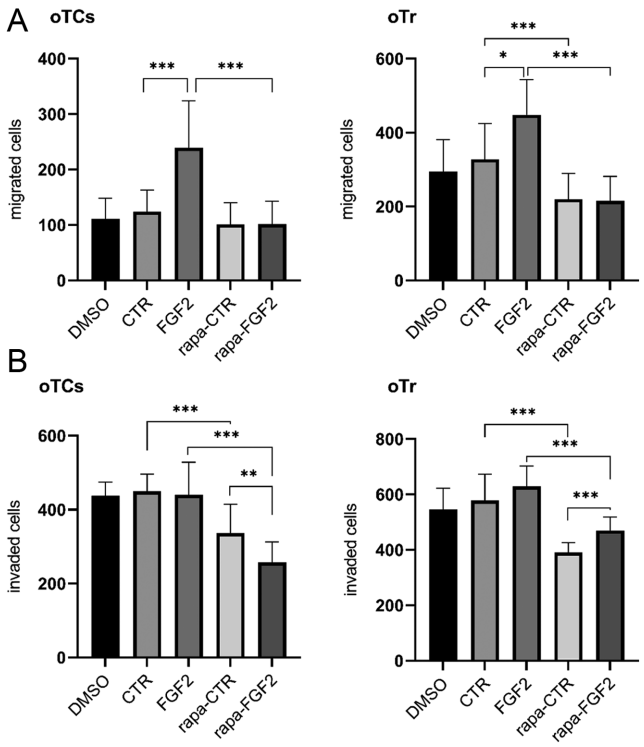

Figure 5 Cell migration and invasion were modulated by the mTOR signalling pathway. Migration and invasion assays were performed by seeding 50,000 cells into the upper chamber of an 8-µm Transwell chamber in serum- and insulin-free DMEM. The Transwell chamber was coated with Matrigel only for invasion assay. oTCs and oTr were subjected to different medium added in the lower chamber according to the treatments. After 12 h of culture, the non-migrated cells were removed, then inserts were fixed and stained with DAPI. Both migration and invasion testes were repeated three different times. Results reported the number of total cells counted after 12 h in 25 random non-overlapping fields. Data are reported as the mean ± standard deviation. One-way analysis of variance was performed (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). oTCs and oTr showed similar results. In normal culture conditions, FGF2 positively influenced cell migration, while mTOR inhibition decreased cell activity (Fig. 5A). FGF2 did not affect cell invasiveness in mTOR-activated culture but, also in this case, mTOR inhibition reduced the number of invaded cells in both systems (Fig. 5B).

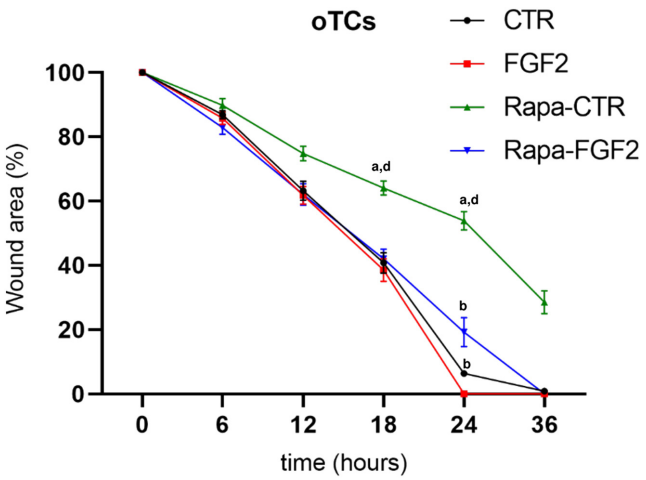

Figure 6 oTC motility was promoted by FGF2 supplementation. Wound-healing scratch assay was done on 12 h starved oTCs 80% confluent by scratching a line across the bottom of the culture dish. About 50 ng/mL FGF2 and 100 nM rapamycin were added to the culture media following treatment conditions, and the cell motility was observed every 6 h. The graph shows the extent of scratch closure obtained under control conditions compared to those with the supplementation. Wound-healing assay was performed three different times for each treatment. mTOR inhibition affects cell motility both with and without FGF2 supplementation compared to their controls. Despite this, motility restoration still seems to be modulated by FGF2 from 12 h. One-way analysis of variance was performed, and data are shown as mean ± S.E.M. Letters a, b, c and d denote differences ($P < 0.05$) vs CTR, FGF2, rapa-CTR and rapa-FG2, respectively.

($P < 0.040$) and *CDH1* ($P < 0.048$) expression, whereas other genes remained mostly unchanged. In these cells, FGF2 supplementation restored *CDH1* ($P < 0.028$) expression, whereas *IFNT* remained downregulated ($P < 0.040$) compared to controls. The expression levels of *FGF2* and its receptor *FGFR2* did not vary under different conditions (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article).

Discussion

This study focused mainly on characterising an *in vitro* culture system of primary cells from sheep placenta to better understand trophoblast adaptation to uterine environmental changes in the early stage of pregnancy. Our findings revealed the role of FGF2 that affects trophoblast response in terms of functionality adaptation through mTOR signalling pathway modulation.

Although previous studies on the basic mechanisms of trophoblast during early placenta growth have been predominantly developed in long-established cell lines (Masters *et al.* 2002), the use of primary cells represents a more physiological tool because morphology, secretion and other functions are better preserved in the tissue context (Pan *et al.* 2009). Even if cell line model gives some advantages, including unlimited cell availability, growth and expansion over longer periods of time, with higher reproducibility of results, primary cells are genetically stable and show healthy cell morphology compared to cell lines in which loss of polarity or lack of key morphology features may develop over time (Unger *et al.* 2002). In particular, cell line may change in phenotype and undergoes functional alteration, whereas the trophoblast primary cells maintain original tissue phenotype and hormone secretory activity (Sullivan 2004), even though for a limited number of passages. Thus, in our study, we focused on 21- to 23-day primary oTCs as a cellular *in vitro* model to explore early placenta development in sheep, by using oTr cell line (Kim *et al.*

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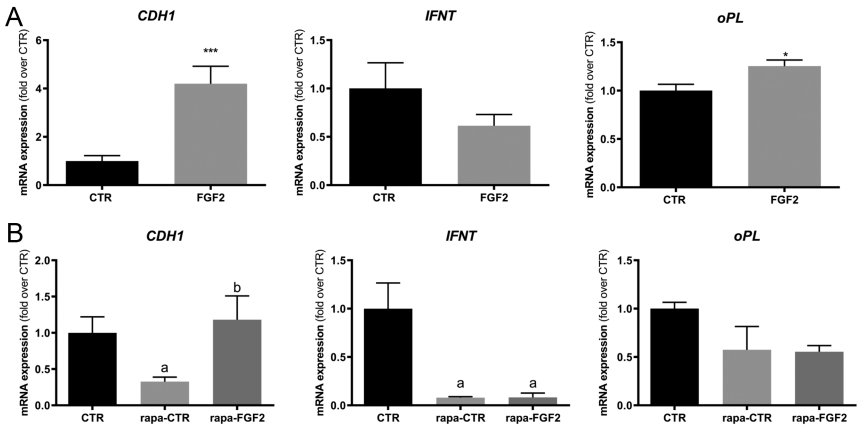

Figure 7 qPCR analysis of genes regulating trophoblast cells function in oTCs. The expression of different genes regulating trophoblast cell function (*CDH1*, *IFNT*, *oPL*) was evaluated after 24 h FGF2 supplementation (Fig.7A), as well as following mTOR inhibition by rapamycin treatment (Fig.7B). RNA was extracted from 24 h oTCs subjected to the different culture conditions. All experiments were repeated three different times, and each qPCR analysis was triplicated. Data were reported as mean ± S.D. (*$P < 0.05$; ***$P < 0.001$). The nonparametric Mann–Whitney U-test (Fig. 7A) and one-way analysis of variance (Fig. 7B) were performed. FGF2 supplementation induced *CDH1* and *oPL* upregulation ($P < 0.001$; $P < 0.05$), whereas no differences were shown for *IFNT* expression (A). Rapamycin-treated cells showed a significant decrease in the expression of *IFNT* ($P < 0.040$) and *CDH1* ($P < 0.048$) (B). In these cells, FGF2 supplementation is able to restore *CDH1* ($P < 0.028$) expression, while *IFNT* remains significantly downregulated ($P < 0.040$) compared to controls (B).

2011) as a supporting tool, mostly in cell functionality approaches.

One of our goal was to better define the cellular model, and the morphological characteristics and gene expression of some typical trophoblast markers were evaluated in oTCs. Primary trophoblast cells and oTr cell line displayed similar phenotype, since the typical mononuclear cluster formation surrounded by bi- and multinuclear cells were observed (Seo *et al.* 2019). Our model showed not only the expression of CK7, which is considered a specific trophoblast marker (Maldonado-Estrada *et al.* 2004), but also the expression of structural (*CDH1*) and hormonal markers (*oPL* and *IFNT*), essential for trophoblast functionality. Furthermore, the typical secretory activity of trophoblast cells was confirmed by progesterone release in the culture medium. The adaptive responses in terms of cell mitogen and migration activity were fairly similar in both models. It is reasonable to suppose that during early placenta development, trophoblasts immediately start to adapt from the end of elongation phase (days 15 and 16) until the firm contact with the maternal endometrium (day 20–23) by increasing proliferation and migration activity.

Notably, several fibroblast growth factors play crucial roles in supporting the successful peri-implantation process in ruminants (Yang *et al.* 2011*a*, Chiuma *et al.* 2020, Devi *et al.* 2020). In particular, FGF2 is considered one of the most relevant growth factors provided by a uterine histotroph in physiological placenta development. In sheep, FGF2 increases from 12 days of pregnancy (Ocón-Grove *et al.* 2008) and is implicated in early conceptus development (Ozawa *et al.* 2013) by promoting the cell proliferative activity of trophoblasts and stimulating cell migration and invasiveness to trigger the syncytialisation process (Pasumarthi *et al.* 1996). In the early stage of sheep placentation (15–23 days following fertilisation), impaired trophoblast proliferation and invasion result in reduced utero-placenta vascularisation, which causes a defective nutrient and oxygen exchange (Hagen *et al.* 2005). Placental insufficiency and fetal growth restriction were often associated with low-FGF2 placental expression, thus compromising pregnancy proceeding (Reynolds & *Redmer* 2001, Fidanza *et al.* 2014).

The real concentration of FGF2 in *in vivo* sheep uteri during pregnancy (Ocòn-Grove *et al.* 2008) remains unclear. Previous studies have examined the effects of different FGF2 concentrations in the trophoblast model, describing 50 ng/mL as the appropriate FGF2 supplementation (Yang *et al.* 2011*b*, Yabe *et al.* 2016, Lim *et al.* 2018). Considering these aspects, we subjected our trophoblast system to 50 ng/mL FGF2 treatment to mimic the FGF2 maternal signal during this crucial developing window of pregnancy.

FGF2 activates several intracellular signalling pathways that mediate diverse cellular functions, such as metabolism, cell growth and survival (Xie *et al.* 2017, Lim *et al.* 2018). MAPK is the most studied signalling pathway during early placenta development in ruminants (Yang *et al.* 2011*a*), whereas only a few studies have reported the role of mTOR as a target in regulating trophoblast activity (Busch *et al.* 2009, Knuth *et al.* 2015, Kim *et al.* 2010). mTOR was proposed as a mechanistic link between uterine nutrient availability

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and conceptus development by promoting biosynthetic pathways, including the synthesis of proteins, lipids and nucleotides (Roos *et al.* 2009, Rabanal-Ruiz *et al.* 2017). The key role of mTOR target in early placenta development was also suggested by *in vitro* outgrowth experiments in which mTOR-deficient blastocysts displayed an aberrant ability to form trophoblasts (Gangloff *et al.* 2004). As shown by western blotting, mTOR signalling pathway activation by FGF2 was also confirmed in our trophoblast system (Fig. 8).

In this study, we explore the effects of FGF2 on cell functionality by mTOR signalling pathway modulation. When the embryo reaches the maximum elongation and closely adheres to endometrium around 21 days of pregnancy in sheep, trophoblast cells are expected to migrate and then partially invade maternal epithelium to establish pregnancy. The exact role for the modulation of mTOR and FGF2 during early placenta development remains uncertain, but our study demonstrates that both molecules are involved in trophoblast cell activity by interacting together.

Findings suggest that mTOR modulates trophoblast functionality and its dysregulation adversely affects all the main studied functions. This could be relevant since the reduced cell migration and invasion are

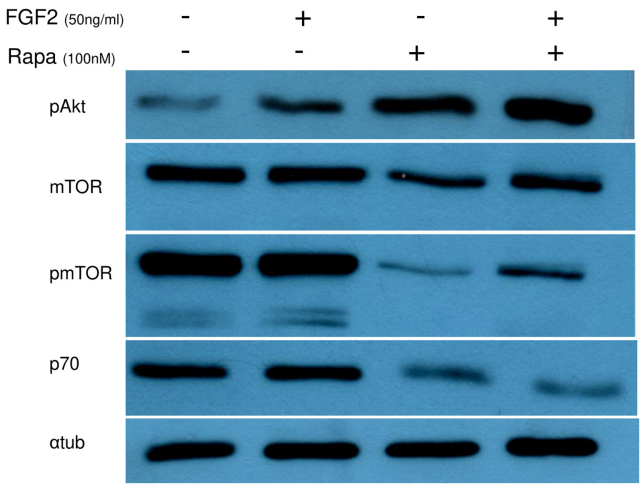

Figure 8 FGF2 affects mTOR signalling pathway activation in oTCs. Serum-starved oTCs were incubated with or without 50 ng/mL FGF2 and 100 nM rapamycin for 24 h. Cells were lysed for 10 min in iced-lysis solution and centrifuged at 4°C for 15 min at 15,000 ×x ***g***. Supernatant was quantified with DC Protein Assays following the manufacture's protocol. About 15 µg protein were resolved on 7.5% polyacrylamide gels and transferred to nitrocellulose blotting membranes. After blocking in 10% BSA, membranes were incubated overnight at 4°C with primary antibodies, washed in TBS–Tween and then incubated 1 h at RT with HRP-conjugated secondary antibodies. Western blot analysis confirmed mTOR activation in CTR and in FGF2 supplementation under normal culture conditions showing mTOR and p70 phosphorylation. On the other hand, in mTOR-inhibited system, FGF2 seemed to phosphorylate mTOR, suggesting that FGF2 was able to restore mTOR activation. αTUB was used as a reference protein.

hallmarks of impaired pregnancy with intrauterine growth restriction that may lead to early pregnancy loss in ruminants. Moreover, we hypothesize that uterine FGF2 could protect trophoblast functionality directly acting on mTOR target, since FGF2 is able to recover mTOR and its downstream activation, as also confirmed by p70 phosphorylation. The specific FGF2 effect on mTOR signalling is also suggested by the expression of e-cadherin. In our study, FGF2 promotes e-cadherin (*CDH1*) upregulation in mTOR-activated system, but it also restores *CDH1* expression even if mTOR is prevented by rapamycin supplementation. On the contrary, previous study demonstrated that FGF2 reduces CDH1 expression during the invasiveness in ovarian cancer cells through the activation of PI3K/Akt/mTOR signalling (Lau *et al.* 2013). During the first trimester of pregnancy, CDH1 downregulation in human trophoblast cells was associated with an increase of motility and invasion activity on implantation side (Shih *et al.* 2022). Low-CDH1 expression is also observed in low-oxygen tension occurring in the early stage of placentation (Arimoto-Ishida *et al.* 2009). In humans, CHD1 loss was associated with the invasion of both cancer and placenta cells, while increasing CDH1 expression in BT-1 cells reflects mature binucleate cells functionality as bovine trophoblast cells invasiveness is limited to the uterine epithelial layer compared to human and rodent placentae (Nakano *et al.* 2005). In the light of these considerations, we conclude that CDH1 expression is dynamically modulated based on differentiation into invading or syncytial trophoblasts, which vary among the species. Moreover, FGF2-mediated *CDH1* upregulation may be associated with an increase of cell–cell interactions in order to guarantee trophoblast cell communication also in mTOR-inhibited system.

Beyond the effect of FGF2-mediated mTOR axis on the structural trophoblast marker, the study revealed the potential impact on key endocrine target genes promoting placenta development.

FGF2 is involved in the maternal recognition signal of pregnancy, as it stimulates IFNT release by the trophectoderm in ruminants (Imakawa *et al.* 2019). This was also confirmed by *in vitro* experiments conducted on bovine CT-1 trophoblast cells (Cooke *et al.* 2009, Yang *et al.* 2011*b*) and blastocysts (Michael *et al.* 2006). However, FGF2 supplementation did not affect IFNT expression in sheep embryos (Moradi *et al.* 2015). A similar result was obtained in our study on oTCs after 1-day treatment, but *IFNT* upregulation was observed after a 4-day treatment, suggesting that the temporal expression pattern of this hormonal release on FGF2 stimulation may differ among ruminants (Supplementary Fig. 1B). Furthermore, our results display that mTOR signalling is involved in *IFNT* expression, since its expression is considerably abolished by rapamycin. It might be suggested that mTOR is a key modulator in placenta development by acting in the signalling cascade

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for the transcription of *IFNT*. Therefore, considering the biological role of IFNT in the implantation signal, mTOR pathway could significantly impact conceptus survival.

Placental lactogen is an early endocrine marker produced by trophoblast cells (Hamlin *et al.* 1994). In sheep, oPL is produced by chorionic binucleate cells at the maternal–fetal interface starting from day 22 (Watkins & Reddy 1980). Rcho-1 trophoblast cells demonstrated that FGF2 stimulated placental lactogen (PL1) mRNA and protein accumulation in a concentration-dependent manner (Peters *et al.* 2000). No previous data are available on the FGF2 effect on placental lactogen in the ruminant placenta. In oTC system, *oPL* was upregulated under FGF2 supplementation; thus, it highlights that FGF2 supports placenta. Overall, we demonstrated that FGF2 affects the expression of structural and hormonal trophoblast markers throughout mTOR signalling. Besides, the results also suggest the pivotal role of mTOR target in trophoblast cell behaviour, given that its prevention causes a severe downregulation of essential markers for conceptus survival, such as IFNT and CDH1.

In summary, this study explores the FGF2-mediated mTOR signalling effect on biological trophoblast functions, including factors involved in pregnancy maintenance. Sheep primary trophoblast cells show that mTOR regulates key cell functions to modulate early placental development. Our findings support the hypothesis that FGF2 operates to modulate trophoblast cell physiology by regulating mTOR axis. In particular, we discovered that mTOR is a key modulator in trophoblast adaptive response, involving the expression of structural and endocrine trophoblast markers, as well as in migration and invasion activity.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-22-0356.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

VI, TP and BM conceived the study. IV, TP, MI and PA performed experiments. IV and TP performed literature search, wrote the paper and analysed data. BM supervisioned the project. TP and BM provided funding.

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