IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1888231 since 2025-01-20T13:36:54Z

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

11 November 2026

IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

This is the author's final version of the contribution published as:

Irene Viola, Paola Toschi, Isabella Manenti, Paolo Accornero and Mario Baratta

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

Reproduction, 165(3), 2024, 313-324. DOI: 10.1530/REP-22-0356.

The publisher's version is available at:
https://rep.bioscientifica.com/view/journals/rep/165/3/REP-22-0356.xml

When citing, please refer to the published version.

Link to this full text:
https://hdl.handle.net/2318/1888231

This full text was downloaded from iris-Aperto: https://iris.unito.it/

iris-AperTO

University of Turin's Institutional Research Information System and Open Access Institutional Repository

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

Viola I.[1], Toschi P.[1]*, Manenti I.[1], Accornero P.[1], Baratta M.[2]

*Corresponding author: paola.toschi@unito.it

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

Key words: sheep, early pregnancy, trophoblast, FGF2, mTOR

Short title: mTOR as key-modulator in sheep placenta

In brief: Fibroblast growth factor-2 (FGF2) is essential for early placenta development in sheep. This study shows as the mechanistic target of rapamycin is 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, 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 foetal–maternal crosstalk.

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.*, 2019) 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), among which is fibroblast growth factor-2 (FGF2) (Ocòn-Grove *et al.*, 2008; Yang *et al.*, 2011a; 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 (IFN-τ) secretion (Yang *et al.*, 2011b).

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' 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); foetal bovine serum (FBS; Gibco, 10270106); L-glutamine (G7513); sodium pyruvate (S8636); non-essential amino acids (Gibco, 11140035); insulin (I9278); FGF-2, rh-FGF-basic (Immunotools, 11343625); trypsin-EDTA (T2601); collagenase-IV (C5138); bovine serum albumin (BSA; A3733); DNase-I (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 (1:1,000, Cell Signalling, 2983), rabbit anti-p-mTOR (1:1,000, Cell Signalling, 5536) and rabbit anti-p-Akt (1:1,000, Cell Signalling, 92715); mouse anti-p-70 (1:1,000, Santa Cruz Biotechnology, sc-8418); mouse anti-cytokeratin-7 (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, Rockford, IL, USA); Alexa-fluor-594 goat-anti-mouse (1:500, Invitrogen, A11032); Alexa-fluor-488 goat-anti-mouse (1:500, Invitrogen, A32723); horseradish peroxidase (HRP)-conjugated secondary antibodies (1:15,000, Immunopure Goat anti-rabbit and anti-mouse IgG, Thermo Fisher Scientific). BrdU (19160), anti-BrdU (1:100, B2531), growth factor-reduced Matrigel Matrix (BD Biosciences, 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 Figure 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 21–23-

day-old placentae (n = 3) were used (Ptak *et al.*, 2013). The uteri were quickly 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 of digestion solution, comprising 25 mg trypsin-EDTA, 25 mg collagenase-IV, 50 mg BSA and 0.2 mg/mL DNase-I 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 1,600 rpm 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 2x was immediately added to equilibrate the osmolarity solution. The solution was centrifuged again at 1,600 rpm for 10 min.

Cells were placed in a Petri dish in trophoblast growth medium, 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 two and eight passages and oTr cell between fourteen and twenty 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 5-bromo-2′-deoxyuridine (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 1,000 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 was 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 10X, 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. 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 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, USA) 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 ~~The~~ cDNA and no-RT samples were used for gene expression analysis using specific 5′–3′ primer pairs designed by Primer3 software (https://primer3.ut.ee) or obtained from previously published papers to anneal at 58/60°C with an amplification efficiency (E) range between 2.1 and 1.9. Cytokeratin-7 (CK7); epithelial-cadherin (CDH1; Seo *et al.*, 2019); ovine placental lactogen (oPL; Lacroix *et al.*, 2002); interferon-tau (IFN-τ; Sakurai *et al.*, 2010); pregnancy-associated glycoprotein (PAG); fibroblast growth factor-2 (FGF2); fibroblast growth factor receptor-2 (FGFR2; McCoski *et al.*, 2018). 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 (ΔΔCT) 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.

End-point PCR was run following the kit's procedure (Qiagen Multiplex PCR Plus Kit, USA). 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 ~~cells~~ 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, 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 x 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, p-Akt mTOR, p-mTOR and p-70. 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 were collected and centrifuged for 15 min at 12,000 rpm 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, 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.

Statistics. 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 t-test was assessed. Data are reported as the mean ± standard deviation. This analysis was used for the wound-healing assays, but data are shown as mean ± standard error of the mean. Cell proliferation was analysed using Fisher's exact test. Statistical differences were considered significant when p was <0.05 in all statistical analyses.

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 sheep embryonic fibroblasts (SEF), which were used as positive and negative controls, respectively (Fig. 3). oTCs and oTr showed the expression of specific trophoblast markers, such as cytokeratin 7 (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 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.

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, IFN-τ and oPL) and FGF2 signalling (FGF2 and FGFR2) were performed in above-mentioned treatments (Fig. 7A-7B). qPCR analyses showed that FGF2 supplementation increased CDH1 ($p < 0.0002$) and oPL ($p < 0.003$) expression, whereas IFN-τ, FGF2 and FGFR2 expression were similar between the control and treated groups. Moreover, rapamycin-treated cells showed a decrease in IFN-τ ($p < 0.040$) and CDH1 ($p < 0.048$) expression, whereas other genes remained mostly unchanged. In these cells, FGF2 supplementation restored CDH1 ($p < 0.028$) expression, whereas IFN-τ remained downregulated ($p < 0.040$) compared to controls. The expression levels of FGF2 and its receptor FGFR2 did not vary under different conditions (Supplementary Figure 1A).

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 (Suillivan, 2003), even though for a limited number of passages. Thus, in our study we focused on 21-23-day primary oTCs as a cellular *in vitro* model to explore early placenta development in sheep, by using oTr cell line (Kim *et al.*, 2011) as a supporting tool, mostly in cell functionality approaches.

One of our goal was to better define the cellular model, 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 the expression of CK7, that is considered a specific trophoblast marker (Maldonado-Estrada *et al.*, 2003), but also the expression of structural (CDH1) and hormonal markers (oPL and IFN-τ), 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 (day 15-16) 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.,* 2011a; Chiuma *et al.,* 2020; Devi *et al.,* 2019). 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.*, 2006). Placental insufficiency and foetal growth restriction were often associated with low-FGF2 placental expression, thus compromising pregnancy proceeding (Reynolds *et al.,* 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 (Yabe *et al.,* 2016; Lim *et al.*, 2018; Yang *et al.,* 2011b). 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.*, 2011a), 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.*, 2019). mTOR was proposed as a mechanistic link between uterine nutrient availability 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 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) up-regulation 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*., 2003). During the first trimester of pregnancy, CDH1 down-regulation 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). Despite CHD1 loss was found to promote the invasion of cancer or human placenta, in BT-1 cells the increase of CDH1 expression reflects mature binucleate cells functionality since bovine trophoblast cells invasiveness is limited to the epithelial layer of uterine caruncles, while in human and rodents' placentae extend to the decidua (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 up-regulation may be associated with an increase of cell-cell interactions in order to guarantee trophoblast cells 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 IFN-τ 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.,* 2011b) and blastocysts (Michael *et al.*, 2006). However, FGF2 supplementation did not affect IFN-τ expression in sheep embryos (Moradi *et al.*, 2015). A similar result was obtained in our study on oTCs after 1 Day-treatment, but IFN-τ 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 Figure 1B). Furthermore, our results display that mTOR signalling is involved in IFN-τ 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 for the transcription of IFN-τ. Therefore, considering the biological role of IFN-τ 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–foetal interface

starting from day 22 (Watkins *et al.*, 1980). Rcho-1 trophoblast cells demonstrated that FGF2 stimulated PL-1 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, ovine placental lactogen 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 down-regulation of essential markers for conceptus survival, such as IFN-τ 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 model displays as mTOR, acting in the main cell functionalities, plays a crucial role in the modulation of early placenta 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

Declaration of interest

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

Funding

This work was supported by Smartsheep project, Fondazione Cassa di Risparmio di Cuneo (CRC), Agricoltura 4.0 and UNITO Grant (TOSP_RILO_20_01).

Author contribution statement

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

References

1. Arimoto-Ishida E, Sakata M, Sawada K, Nakayama M, Nishimoto F, Mabuchi S, Takeda T, Yamamoto T, Isobe A, Okamoto Y, Lengyel E, Suehara N, Morishige K & Kimura T 2009. Up-

regulation of alpha5-integrin by E-cadherin loss in hypoxia and its key role in the migration of extravillous trophoblast cells during early implantation. *Endocrinology* **150** (9) 4306-4315.

2. Bazer FW, Wu G, Johnson GA, Kim J & Song G 2011. Uterine histotroph and conceptus development: select nutrients and secreted phosphoprotein 1 affect mechanistic target of rapamycin cell signaling in ewes. *Biology of Reproduction* **85** (6) 1094-1107.

3. Bazer FW, Wang X, Johnson GA & Wu G 2015. Select nutrients and their effects on conceptus development in mammals. *Animal Nutrition* **1** (3) 85-95.

4. Belkacemi L, Nelson DM, Desai M & Ross MG 2010. Maternal undernutrition influences placental-fetal development. *Biology of Reproduction* **83** (3) 325-31.

5. Bonometti S, Menarim BC, Reinholt BM, Ealy AD & Johnson SE 2019. Growth factor modulation of equine trophoblast mitosis and prostaglandin gene expression. *Journal of Animal* Science **97** (2) 865-873.

6. Busch S, Renaud SJ, Schleussner E, Graham CH & Markert UR 2009. mTOR mediates human trophoblast invasion through regulation of matrix-remodeling enzymes and is associated with serine phosphorylation of STAT3. *Experimental Cell Research* 10 **315** (10) 1724-1733.

7. Chiumia D, Schulke K, Groebner AE, Waldschmitt N, Reichenbach HD, Zakhartchenko V, Bauersachs S & Ulbrich SE 2020. Initiation of Conceptus Elongation Coincides with an Endometrium Basic Fibroblast Growth Factor (FGF2) Protein Increase in Heifers. *International Journal of Molecular Sciences* **26** 21(5) 1584.

8. Cooke FN, Pennington KA, Yang Q & Ealy AD 2009. Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm. *Reproduction* **137** (2) 259-69.

9. Chu A, Thamotharan S, Ganguly A, Wadehra M, Pellegrini M & Devaskar SU 2016. Gestational food restriction decreases placental interleukin-10 expression and markers of autophagy and endoplasmic reticulum stress in murine intrauterine growth restriction. *Nutrition Research* **36** (10)1055-1067.

10. Devi HL, Kumar S, Konyak YY, Bharati J, Bhimte A, Pandey Y, Kumar KV, Paul A, Kala A, Samad, HA, Verma MR, Singh G, Bag S, Sarkar M & Chouhan VS 2019. Expression and functional role of fibroblast growth factors (FGF) in placenta during different stages of pregnancy in water buffalo (Bubalus bubalis). *Theriogenology* **143** 98-112.

11. Fidanza A, Toschi P, Zacchini F, Czernik M, Palmieri C, Scapolo P, Modlinski JA, Loi P & Ptak GE 2014. Impaired placental vasculogenesis compromises the growth of sheep embryos developed in vitro. *Biology of Reproduction* **91** (1) 21.

12. Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G & Kozma SC 2004. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Molecular and Cell Biology* **24** (21) 9508-9516.

13. Grazul-Bilska AT, Borowicz PP, Johnson ML, Minten MA, Bilski JJ, Wroblewski R, Redmer DA & Reynolds LP 2010. Placental development during early pregnancy in sheep: vascular growth and expression of angiogenic factors in maternal placenta. *Reproduction* **140** (1) 165-174.

14. Gupta MB & Jansson T 2019. Novel roles of mechanistic target of rapamycin signaling in regulating fetal growth. *Biology of Reproduction* **100** (4)872-884.

15. Hagen AS, Orbus RJ, Wilkening RB, Regnault TR & Anthony RV 2006. Placental expression of angiopoietin-1, angiopoietin-2 and tie-2 during placental development in an ovine model of placental insufficiency-fetal growth restriction. *Pediatric Research* **58** (6)1228-32.

16. Hamlin GP, Lu XJ, Roby KF & Soares MJ 1994. Recapitulation of the pathway for trophoblast giant cell differentiation in vitro: stage-specific expression of members of the prolactin gene family. *Endocrinology* **134** 2390–2396.

17. Igwebuike UM 2006 Trophoblast cells of ruminant placentas-A minireview. *Animal Reproduction Science* **93** (3–4) 185-198.

18. Imakawa K, Kusama K & Jiro Y 2019. Early placentation and local immune regulation. *Bioscientifica Proceedings* **8.**

19. Johnson GA, Bazer FW, Burghardt RC, Wu G, Seo H, Kramer AC & McLendon BA 2018 Cellular events during ovine implantation and impact for gestation. *Animal reproduction* **15** 843-855.

20. Kim JY, Burghardt RC, Wu G, Johnson GA, Spencer TE & Bazer FW 2011. Select nutrients in the ovine uterine lumen. VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. *Biology of Reproduction* **84** (1) 62-69.

21. Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, Johnson GA & Bazer FW 2019. Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. *Matrix Biology* **29** (5) 369-382.

22. Knuth A, Liu L, Nielsen H, Merril D, Torry DS & Arroyo JA 2015. Placenta growth factor induces invasion and activates p70 during rapamycin treatment in trophoblast cells. *American Journal of Reproductive Immunology* **73** (4) 330-340.

529 23. Lacroix MC, Bolifraud P, Durieux D, Pauloin A, Vidaud M & Kann G 2022. Placental Growth
530 Hormone and Lactogen Production by Perifused Ovine Placental Explants: Regulation by Growth
531 Hormone-Releasing Hormone and Glucose. Biology of *Reproduction* **66** (3) 555–561.

532 24. Lau MT, So WK & Leung PC 2013. Fibroblast growth factor 2 induces E-cadherin down-
533 regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells. *PLoS One* **8** (3)
534 e59083.

535 25. Lim W, Bae H, Bazer FW & Song G 2018. Fibroblast growth factor 2 induces proliferation and
536 distribution of G_2/M phase of bovine endometrial cells involving activation of PI3K/AKT and MAPK
537 cell signaling and prevention of effects of ER stress. *Journal of Cellular Physiology* **233** (4) 3295-
538 3305.

539 26. Lin X, Zhang Y, Liu L, McKeehan WL, Shen Y, Song S & Wang F 2011. FRS2α is essential for
540 the fibroblast growth factor to regulate the mTOR pathway and autophagy in mouse embryonic
541 fibroblasts. *International Journal of Biological Sciences* **7** (8) 114-121.

542 27. Masters JR 2002. HeLa cells 50 years on: the good, the bad and the ugly. *Nature Reviews*
543 *Cancer* **2** (4) 315-319.

544 28. Michael DD, Alvarez IM, Ocón OM, Powell AM, Talbot NC, Johnson SE & Ealy AD 2006.
545 Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau
546 production in bovine trophectoderm. *Endocrinology* **147** (7) 3571-9.

547 28. McCoski SR, Poole RK, Vailes McCauley T & Ealy AD 2018. Maternal obesity alters the
548 expression of embryonic regulatory transcripts in the preimplantation ovine conceptus.
549 *Reproductive Biology* **18** (2) 198-201. 29. Moradi M, Riasi A, Ostadhosseini S, Hajian M, Hosseini
550 M, Hosseinnia P & Nasr-Esfahani MH 2015. Expression profile of FGF receptors in preimplantation
551 ovine embryos and the effect of FGF2 and PD173074. *Growth Factors* **33** (5-6) 393-400.

552 30. Maldonado-Estrada J, Menu E, Roques P, Barré-Sinoussi F & Chaouat G 2004. Evaluation of
553 Cytokeratin 7 as an accurate intracellular marker with which to assess the purity of human placental
554 villous trophoblast cells by flow cytometry. *Journal of Immunology Methods* **286** (1-2) 21-34.

555 31. Nakano H, Shimada A, Imai K, Takahashi T & Hashizume K 2005. The cytoplasmic expression
556 of E-cadherin and β-catenin in bovine trophoblasts during binucleate cell differentiation. *Placenta*
557 **26** (5) 394-401.

558 32. Ocón-Grove OM, Cooke FN, Alvarez IM, Johnson SE, Ott TL & Ealy AD 2008. Ovine
559 endometrial expression of fibroblast growth factor (FGF) 2 and conceptus expression of FGF
560 receptors during early pregnancy. *Domestic animal endocrinology* **34** *(2)* 135-45.

33. Ozawa M, Yang QE & Ealy AD 2013. The expression of fibroblast growth factor receptors during early bovine conceptus development and pharmacological analysis of their actions on trophoblast growth in vitro. *Reproduction* **24** 145(2) 191-201.

34. Pan C, Kumar C, Bohl S, Klingmueller U, Mann M 2009. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. *Molecular & Cellular Proteomics* **8** (3) 443-450.

35. Pasumarthi KB, Kardami E & Cattini PA 1996. High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine and intracrine actions of fibroblast growth factor-2. *Circulation Research* **78** (1) 126-136.

36. Peters TJ, Chapman BM, Wolfe MW & Soares MJ 2000. Placental lactogen-I gene activation in differentiating trophoblast cells: extrinsic and intrinsic regulation involving mitogen-activated protein kinase signaling pathways. *Journal of Endocrinology* **165** (2) 443-456.

37. Pfarrer C, Weise S, Berisha B, Schams D, Leiser R, Hoffmann B & Schuler G 2006. Fibroblast growth factor (FGF)-1, FGF2, FGF7 and FGF receptors are uniformly expressed in trophoblast giant cells during restricted trophoblast invasion in cows. *Placenta* **27** (6-7) 758-770.

38. Pijuan J, Barceló C, Moreno DF, Maiques O, Sisó P, Marti RM, Macià A & Panosa A 2019. *In vitro* Cell Migration, Invasion, and Adhesion Assays: From Cell Imaging to Data Analysis. *Frontiers in Cell and Developmental Bioloogy* **14** 7:107.

39. Pringle KG, Kind KL, Sferruzzi-Perri AN, Thompson JG & Roberts CT 2010. Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. *Human Reproduction Update* **16** (4) 415-431.

40. Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M, Monaco F & Loi P 2013. Post-implantation mortality of in vitro produced embryos is associated with DNA methyltransferase 1 dysfunction in sheep placenta. *Human Reproduction* **28** (2) 298-305.

41. Rabanal-Ruiz Y, Otten EG & Korolchuk VI 2017. mTORC1 as the main gateway to autophagy. *Essays in Biochemistry*. 12 **61** (6) 565-584.

42. Reynolds LP & Redmer DA 2001. Angiogenesis in the placenta. *Biology of Reproduction*. **64** (4) 1033-1040.

43. Unger RE, Krump-Konvalinkova V, Peters K & Kirkpatrick CJ 2002. In Vitro Expression of the Endothelial Phenotype: Comparative Study of Primary Isolated Cells and Cell Lines, Including the Novel Cell Line HPMEC-ST1.6R. *Microvascular Research* **64** (3), 384-397.

593 44. Roos S, Powell TL & Jansson T 2009. Placental mTOR links maternal nutrient availability to
594 fetal growth. *Biochemical Society Transactions*. **37** (1) 295-8.

595 45. Sakurai T, Bai H, Konno T, Ideta A, Aoyagi Y, Godkin JD & Imakawa K 2010. Function of a
596 Transcription Factor CDX2 Beyond Its Trophectoderm Lineage Specification, *Endocrinology* 151
597 (12) 5873–5881.

598 46. Seo H, Bazer FW, Burghardt RC & Johnson GA 2019. Immunohistochemical Examination of
599 Trophoblast Syncytialization during Early Placentation in Sheep. *Internation Journal of Molecular*
600 *Science* **13** 20 (18):4530.

601 47. Shih IM, Hsu MY, Oldt RJ, Herlyn M, Gearhart JD & Kurman JR 2022. The Role of E-cadherin
602 in the Motility and Invasion of Implantation Site Intermediate Trophoblast. *Placenta* **23** (10) 706-
603 715.48. Spencer TE, Johnson GA, Bazer FW & Burghardt R 2004 Implantation mechanisms:
604 insights from the sheep. *Reproduction* **128** (6) 657-668.

605 49. Suarez-Arnedo A, Torres Figueroa F, Clavijo C, Arbeláez P, Cruz JC & Muñoz-Camargo C
606 2020. An image J plugin for the high throughput image analysis of in vitro scratch wound healing
607 assays. *PLoS One* **28** 15 (7) e0232565.

608 50. Sullivan MHF 2004. Endocrine cell lines from the placenta. *Molecular and Cellular*
609 *Endocrinology* **228** (1–2) 103-119.

610 51. Toschi P & Baratta M 2021. Ruminant Placental Adaptation in Early Maternal Undernutrition:
611 An Overview. *Frontiers in Veterinary Science* **20** (8) 755034.

612 52. Wang X, Wu G & Bazer FW 2016. mTOR: The Master Regulator of Conceptus Development in
613 Response to Uterine Histotroph During Pregnancy in Ungulates. In *Molecules to Medicine with*
614 *mTOR,* pp 23-35. Eds Kenneth Maiese. Academic Press.

615 53. Wang Y, Pan XF, Liu GD, Liu ZH, Zhang C, Chen T & Wang YH 2021. FGF-2 suppresses
616 neuronal autophagy by regulating the PI3K/Akt pathway in subarachnoid hemorrhage. *Brain*
617 *Research Bulletin* **173** 132-140.

618 54. Watkins WB & Reddy S 1980. Ovine placental lactogen in the cotyledonary and
619 intercotyledonary placenta of the ewe. *Journal of Reproduction and Fertility* **58** (2) 411-414.

620 55. Wen HY, Abbasi S, Kellems RE & Xia Y 2005. mTOR: a placental growth signaling sensor.
621 *Placenta* **26** (A) S63-69.

622 56. Xie M, McCoski SR, Johnson SE, Rhoads ML & Ealy AD 2017. Combinatorial effects of
623 epidermal growth factor, fibroblast growth factor 2 and insulin-like growth factor 1 on trophoblast
624 cell proliferation and embryogenesis in cattle. *Reproduction Fertility and Development* **29** (2) 419-
625 430.

57. Yang QE, Giassetti MI & Ealy AD 2011a. Fibroblast growth factors activate mitogen-activated protein kinase pathways to promote migration in ovine trophoblast cells. *Reproduction* **141** *(5)* 707-714.

58. Yang QE, Johnson SE & Ealy AD 2011b. Protein kinase C delta mediates fibroblast growth factor-2-induced interferon-tau expression in bovine trophoblast. *Biology of Reproduction* **84** (5) 933-43.

59. Yabe S, Alexenko AP, Amita M, Yang Y, Schust DJ, Sadovsky Y, Ezashi T & Roberts RM 2016. Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas. *Proceedings of the National Acadademy of Sciences USA*. 10 **113** (19) E2598-2607.

Legends

Fig 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 21-23 Day 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. Firstly, oTCs and oTr phenotype was observed by cell microscopy for 7 days to follow the typical trophoblast cell differentiation in each culture models. Then, trophoblast marker detection was performed by immunofluorescence cell staining, end-point PCR and immunoenzymatically 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.

Fig 2. oTCs primary cells and oTr cell line morphology. 21-23 days 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 ~~mononuclear~~ “cobblestone-clusters" (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: 5x magnification; C, D, G, H: 10x magnification. Scalebar at both magnifications: 100 µm.

Fig 3. oTCs characterization. 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 Cytokeratin-7 (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 (20x magnification; scalebar: 100 µm). Conventional PCR (G) was conducted three times on oTCs in order to show structural and hormonal trophoblast marker genes. oTCs display ~~showing~~ a Cytocheratin-7 (CK7), e-cadherin (CDH1), Interferon-τ (IFN-τ), 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 centrifuge 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 time. Both cellular models showed progesterone release in their growth medium.

Fig 4. FGF2 effect on proliferation of oTCs and oTr. Cells were seeded in 4-well chamber slides, starved at 60% of confluence for 24h 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. 1,000 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 was analysed by Fisher’s exact test (*$p < 0.05$; ****$p < 0.0001$). The graphs relieve that FGF2 increased cellular mitogen activity of both oTCs and oTr starting from 24 h.

Fig 54. Cell migration and invasion were modulated by the mTOR signalling pathway.

Migration and Invasion assays were performed seeding. 50,000 cells into the upper chamber of an 8-µm Transwell chamber in serum- and insulin-free DMEM. Transwell were 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).

Fig 6. oTCs 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. 50 ng/ml FGF2 and 100nM rapamycin were added to the culture media following treatments 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 12h. One-way analysis of variance was performed and data are shown as mean ± standard error of the mean. Letter a,b, c and d denote differences ($p < 0.05$) vs CTR, FGF2, rapa-CTR and rapa-FG2, respectively.

Fig 7. qPCR analysis of genes regulating trophoblast cells function in oTCs. Expression of different genes regulating trophoblast cells function (CDH1, IFN-τ, oPL) were evaluated after 24h FGF2 supplementation (Fig.6A), 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 ± standard deviation (*$p < 0.05$; *** $p < 0.001$). The nonparametric Mann–Whitney t-test (Fig. 7A) and one-way analysis of variance (Fig. 7B) were performed. FGF2 supplementation induced CDH1 and oPL up-regulation ($p < 0.001$; $p < 0.05$), whereas no differences were shown for IFN-τ expression (Fig. 7A). Rapamycin treated-cells showed a significant decrease in the expression of IFN-τ ($p < 0.040$) and CDH1 ($p < 0.048$) (Fig. 7B). In these cells FGF2 supplementation is able to restore CDH1 ($p < 0.028$) expression

while IFN-τ remains significantly downregulated ($p < 0.040$) compared to controls (Fig. 7B).

Fig 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. 15 µg protein was resolved on 7.5% polyacrylamide gels and transfer 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 1h 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 phosphorylated mTOR, suggesting that FGF2 was able to restore mTOR activation. α-TUB was used as a reference protein.

Figure 1.

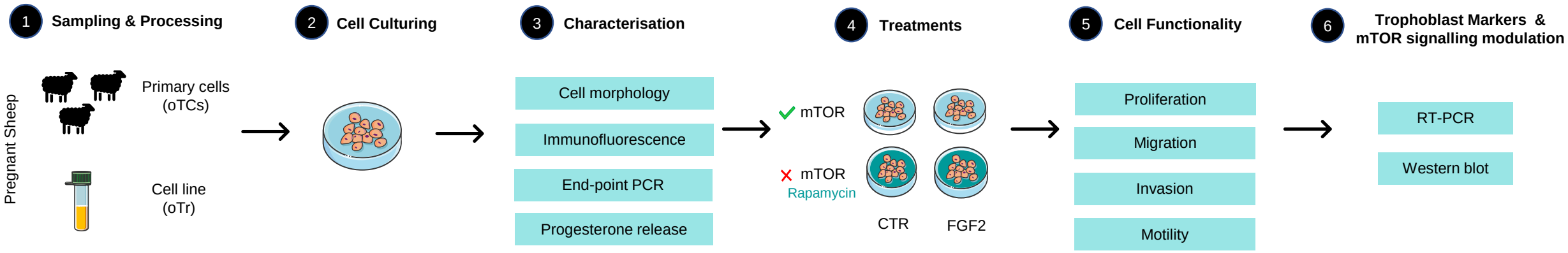

1 Sampling & Processing
2 Cell Culturing
3 Characterisation
4 Treatments
5 Cell Functionality
6 Trophoblast Markers & mTOR signalling modulation
Pregnant Sheep
Primary cells (oTCs)
Cell line (oTr)
Cell morphology
Immunofluorescence
End-point PCR
Progesterone release
mTOR
mTOR Rapamycin
CTR
FGF2
Proliferation
Migration
Invasion
Motility
RT-PCR
Western blot

Figure 2.

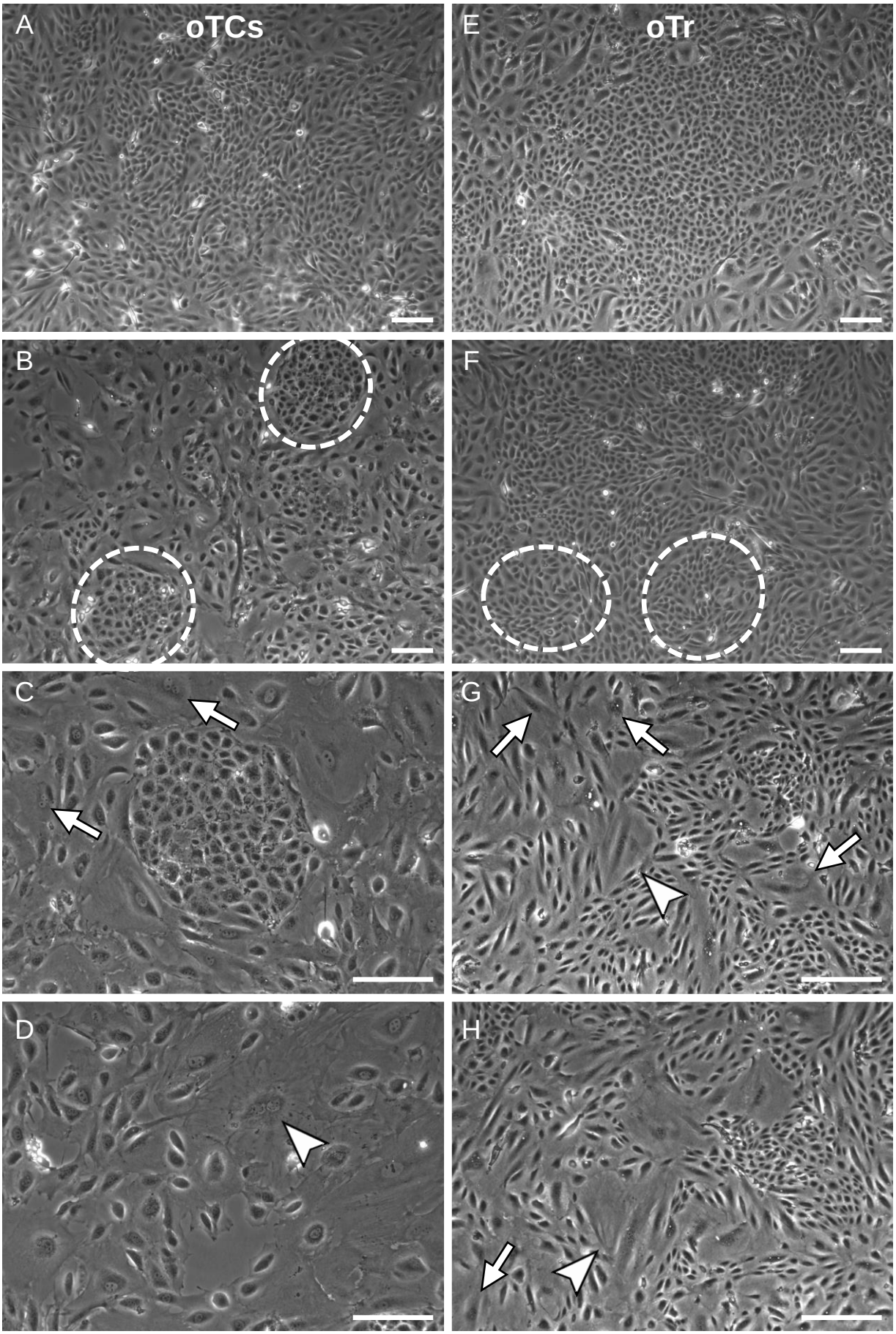
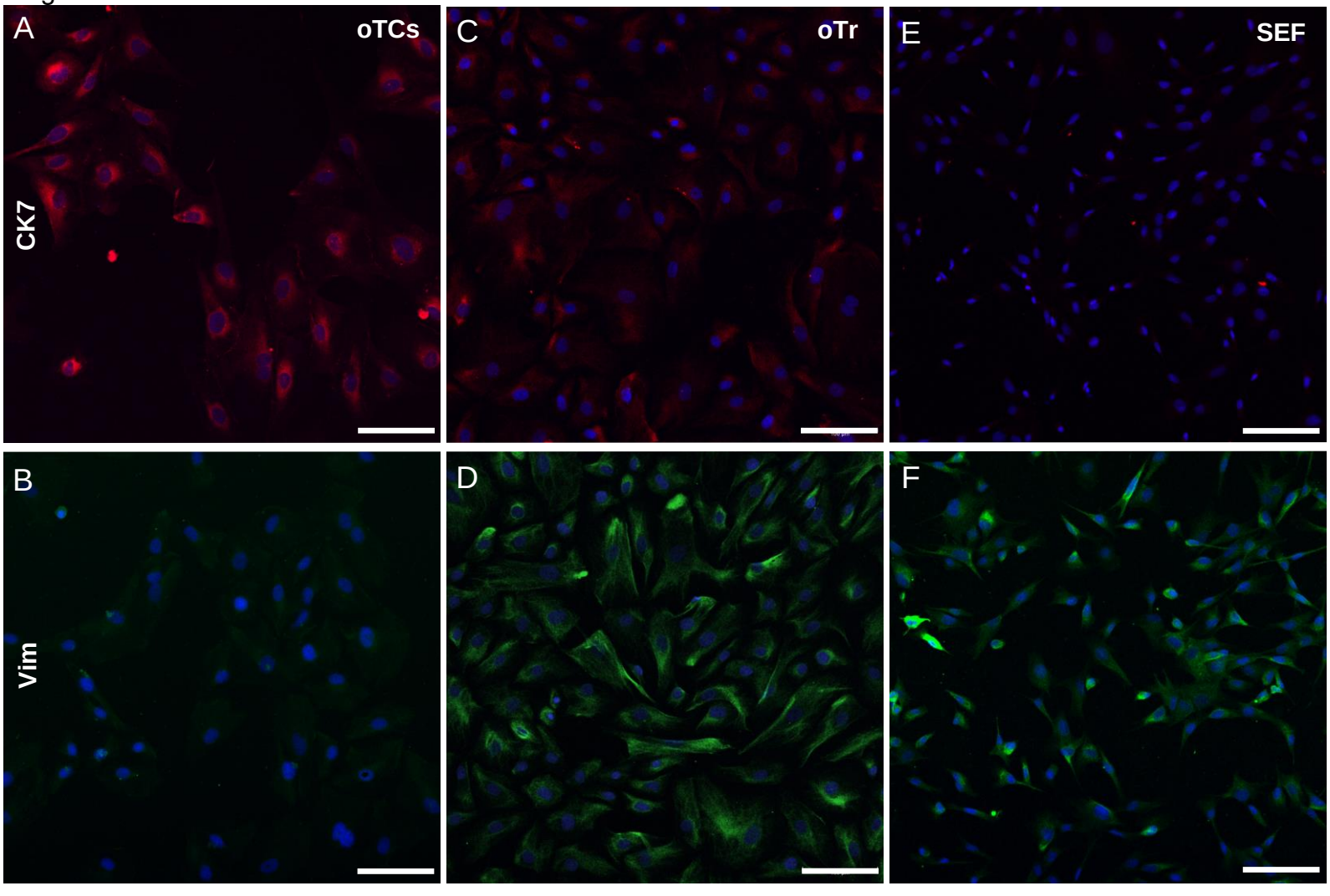
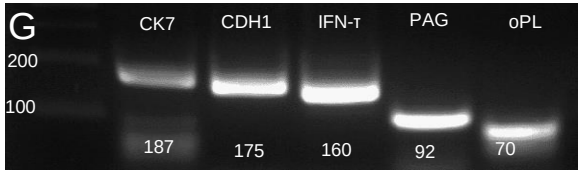


Figure 3.


A
oTCs
CK7
C
oTr
E
SEF
B
Vim
D
F


G
CK7
CDH1
IFN-τ
PAG
oPL
200
100
187
175
160
92
70

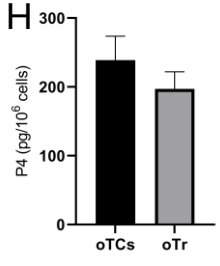

H
300
200
100
0
P4 (pg/10^6 cells)
oTCs
oTr

Figure 4.

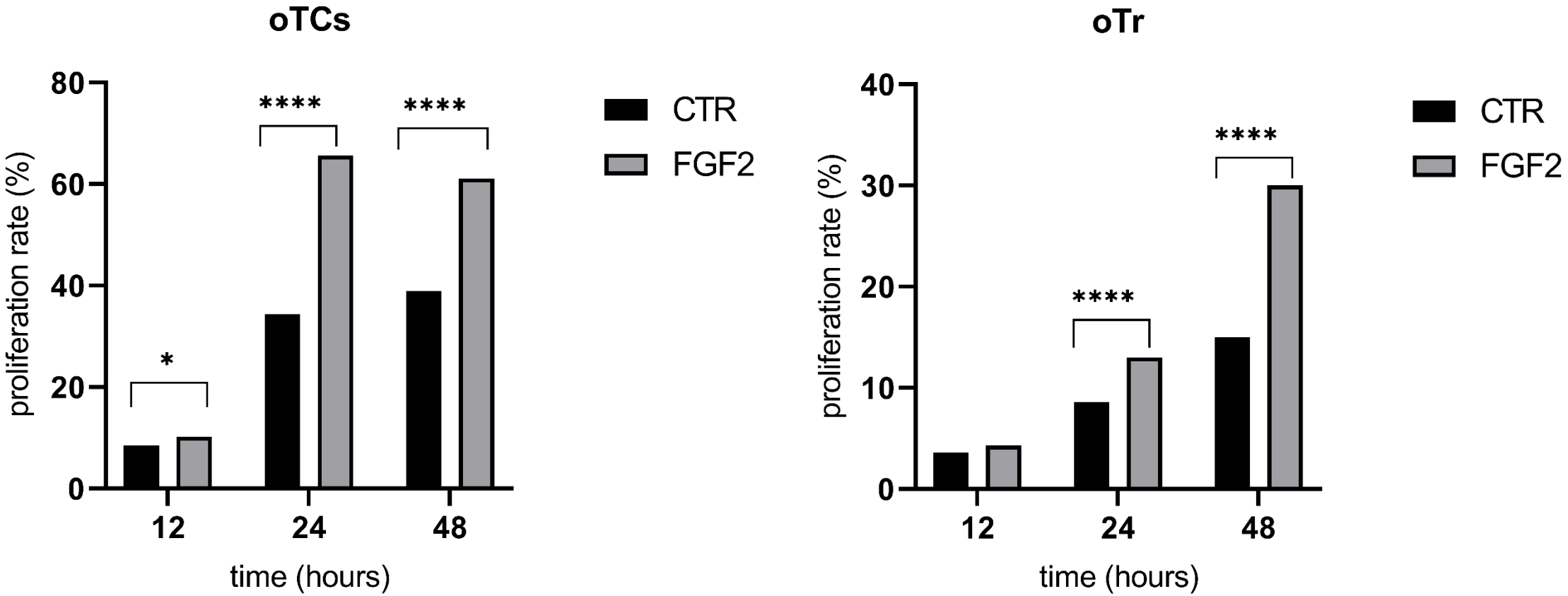

oTCs
proliferation rate (%)
80
60
40
20
0
*
****
****
12
24
48
time (hours)
CTR
FGF2
oTr
proliferation rate (%)
40
30
20
10
0
****
****
12
24
48
time (hours)
CTR
FGF2

Figure 5.

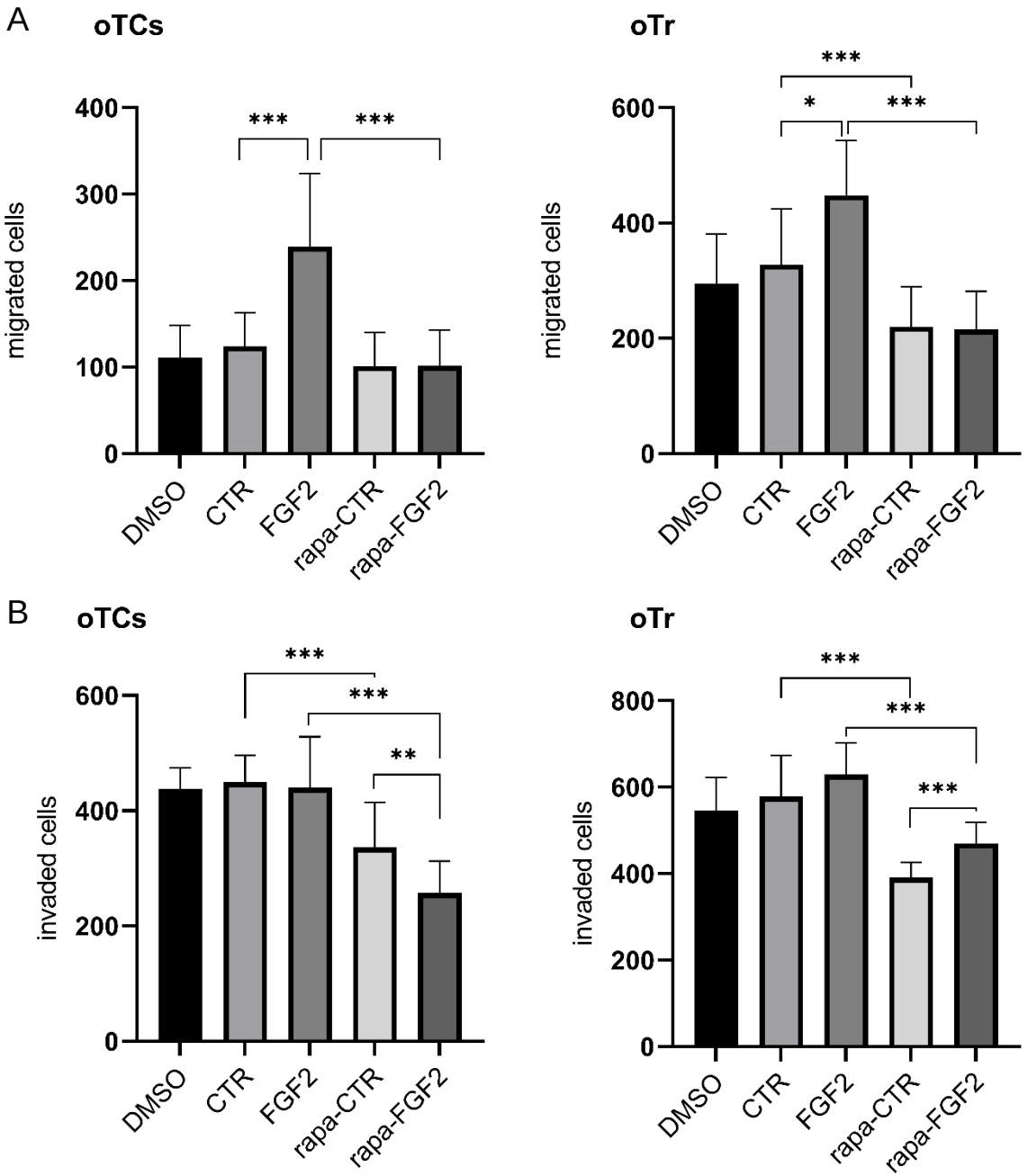

A
oTCs
migrated cells
400
300
200
100
0
***
***
DMSO
CTR
FGF2
rapa-CTR
rapa-FGF2
oTr
migrated cells
600
400
200
0
***
*
***
DMSO
CTR
FGF2
rapa-CTR
rapa-FGF2
B
oTCs
invaded cells
600
400
200
0
***
***
**
DMSO
CTR
FGF2
rapa-CTR
rapa-FGF2
oTr
invaded cells
800
600
400
200
0
***
***
***
DMSO
CTR
FGF2
rapa-CTR
rapa-FGF2

Figure 6.

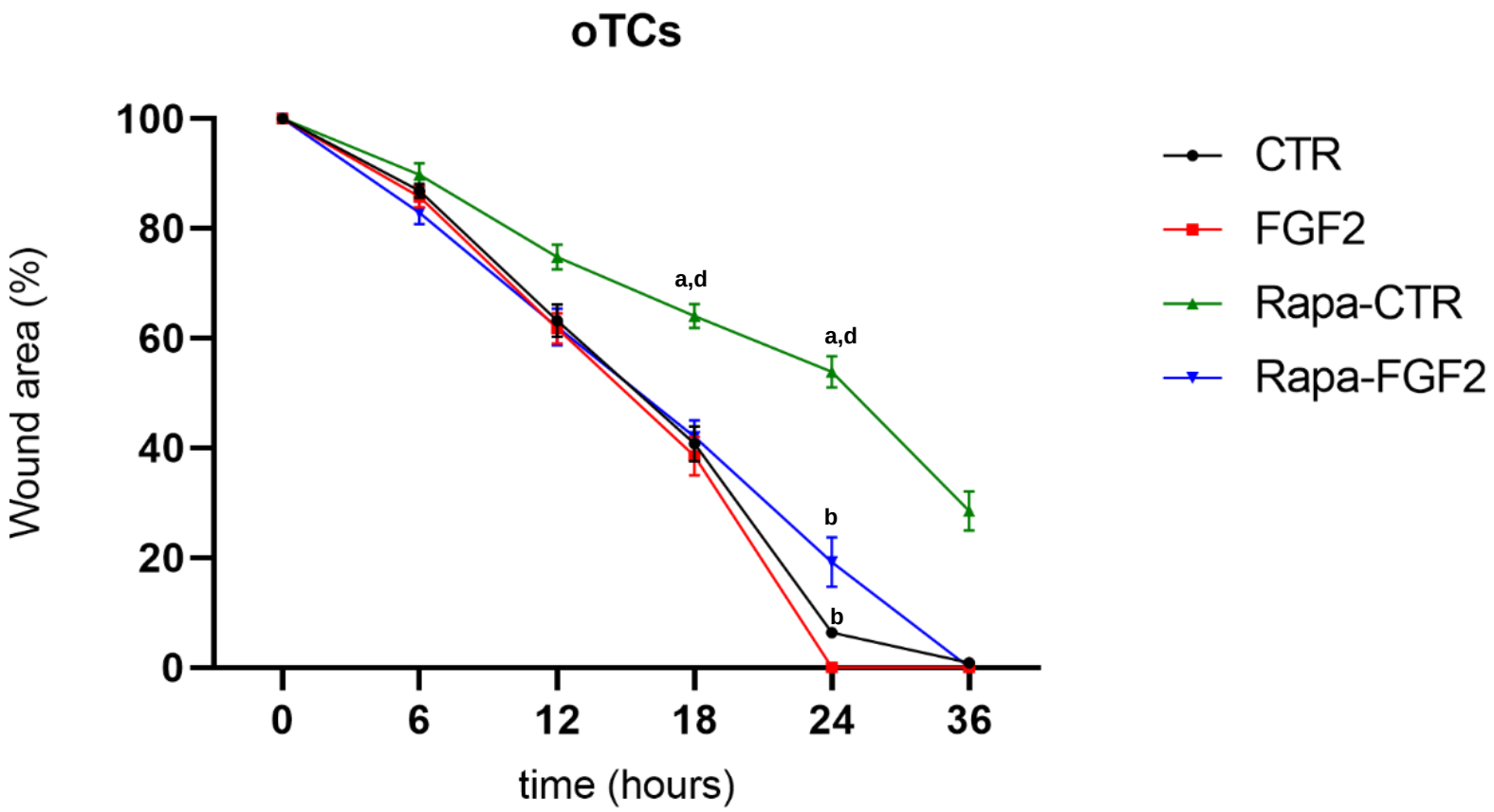
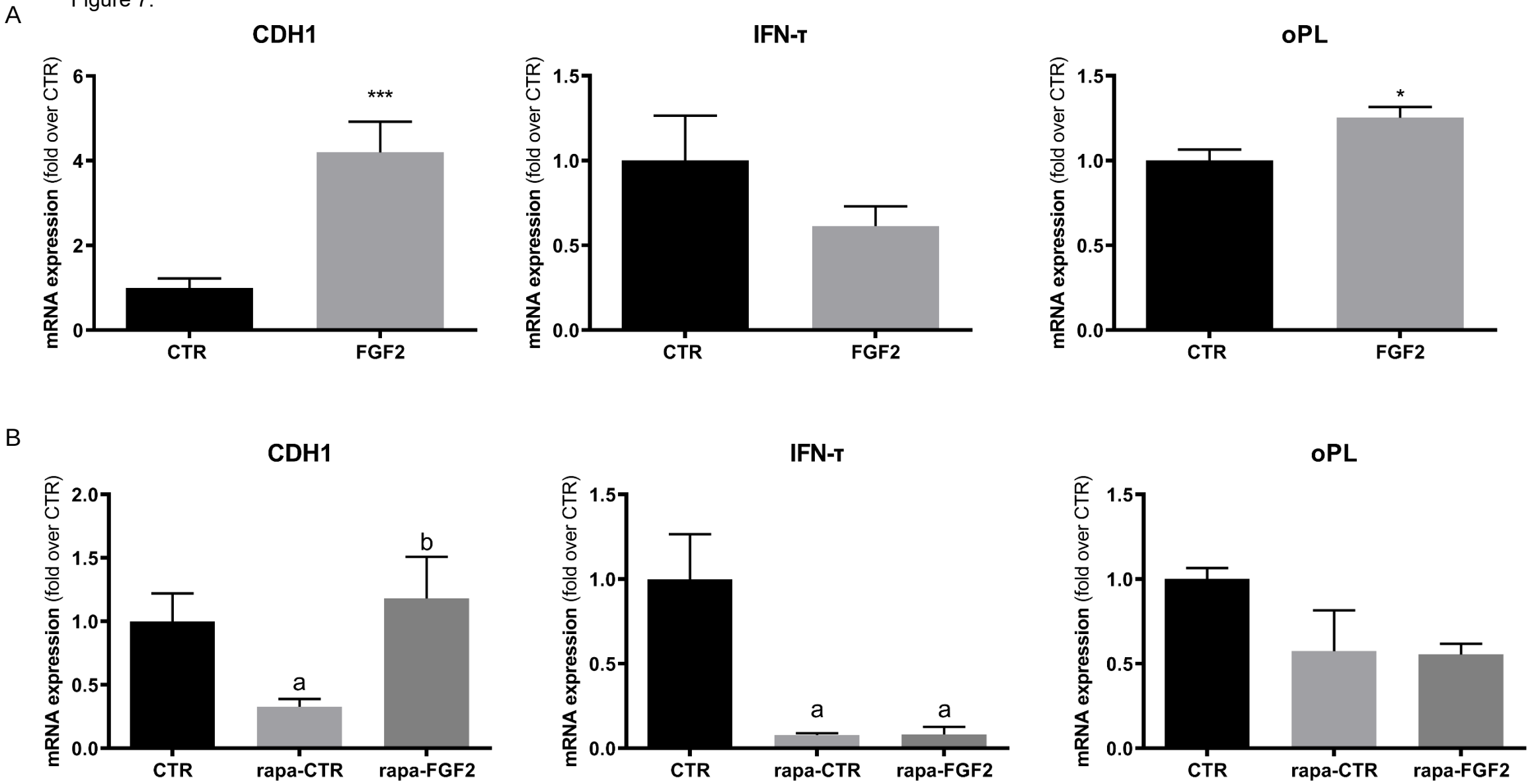

oTCs
Wound area (%)
100
80
60
40
20
0
0
6
12
18
24
36
time (hours)
a,d
a,d
b
b
CTR
FGF2
Rapa-CTR
Rapa-FGF2

Figure 7.


A
CDH1
IFN-τ
oPL
mRNA expression (fold over CTR)
***
*
CTR
FGF2
B
CDH1
IFN-τ
oPL
a
b
rapa-CTR
rapa-FGF2

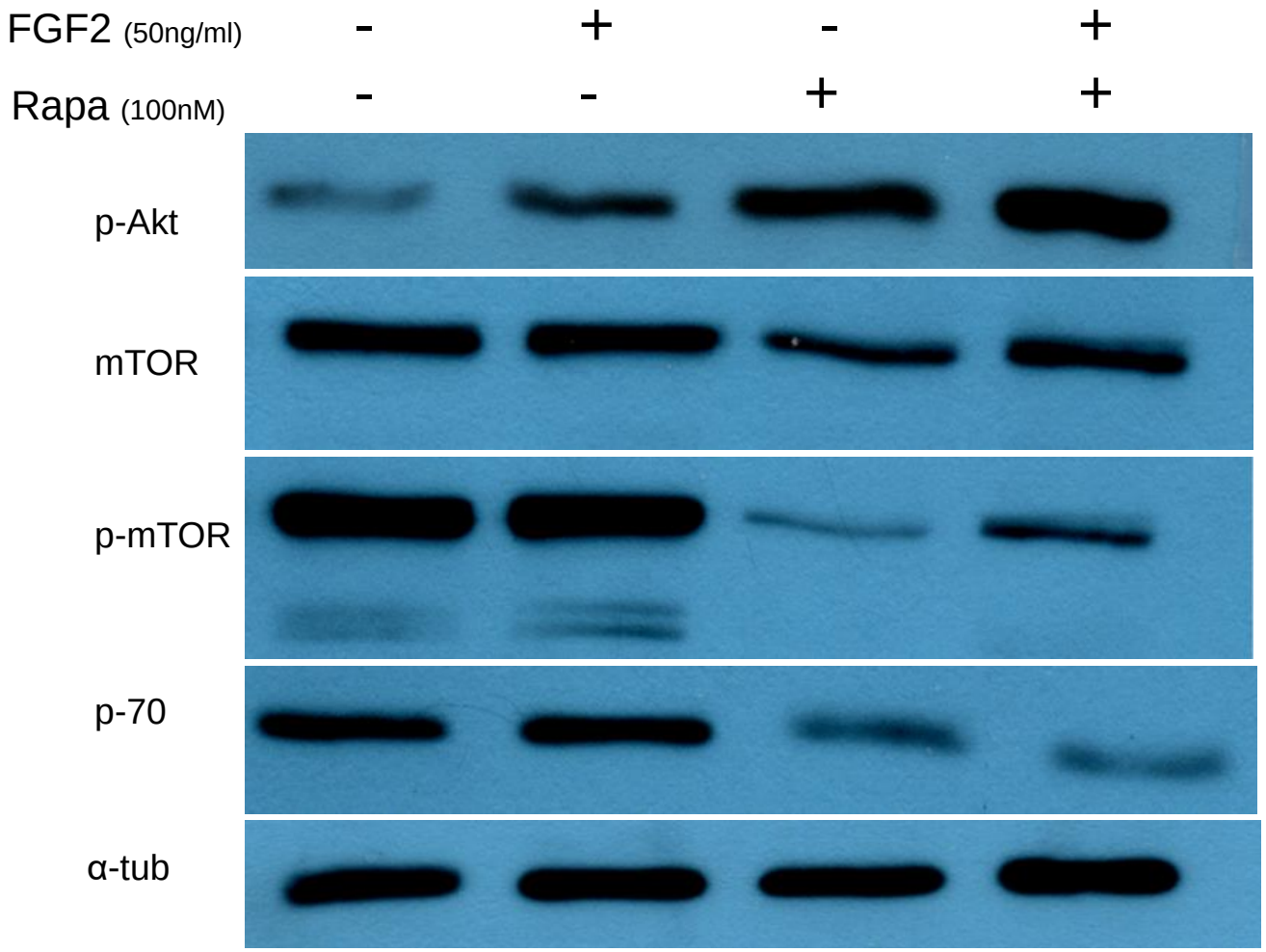



Figure 8.