Fibroblast growth factors activate mitogen-activated protein kinase pathways to promote migration in ovine trophoblast cells

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Abstract

Fibroblast growth factors (FGFs) 2 and FGF10 are uterine- and conceptus-derived factors that mediate trophoblast activities in cattle and sheep. To extend our understanding of how FGFs may control peri-implantation development in ruminants, we determined whether FGF2 and FGF10 impact trophoblast cell migration. Transwell inserts containing 8 μm pores were used to examine whether FGF2 or FGF10 supplementation increased oTr1 cell migration. Supplementation with 0.5 ng/ml FGF2 or FGF10 did not affect oTr1 cell migration number, but exposure to 5 or 50 ng/ml FGF2 or FGF10 increased (P<0.05) oTr1 cell migration when compared with controls. The involvement of specific MAP kinase (MAPK) cascades in mediating this FGF response was examined by using pharmacological inhibitors of specific MAPKs. Western blot analysis indicated that FGF2 and FGF10 increased phosphorylation status of MAPKs 1, 3, 8, 9, and 14. Exposure to specific inhibitors blocked FGF induction of each MAPK. Exposure to inhibitors before supplementation with FGF2 or FGF10 prevented FGF induction of cell migration, indicating that each of these signaling molecules was required for FGF effects. A final series of studies examined whether FGF2 and FGF10 also mediated the migration of a bovine trophoblast line (CT1 cell). Increases in migration were detected in each cell line by supplementing 5 or 50 ng/ml FGF2 or FGF10 (P<0.05). In summary, FGF2 and FGF10 regulate migratory activity of ovine trophoblast cells through MAPK-dependent pathways. These outcomes provide further evidence that FGFs function as mediators of peri-implantation conceptus development in cattle and sheep.

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Introduction

Substantial conceptus development occurs in cattle, sheep, and other ruminants prior to uterine adhesion and implantation. Unlike rodents and primates, where conceptuses begin implanting soon after hatching from the zona pellucida, ruminant conceptuses remain free-floating for an extended period. In cattle, trophoblast adhesion to the uterine lining is not evident until days 19–21 of pregnancy (Guillomot 1995). Extensive morphological changes in the conceptus occur prior to uterine attachment. Gastrulation and neurulation occur within the epiblast during this period. Notable changes also occur within the trophectoderm. Around days 12–13 in sheep and days 14–16 in cattle, a combination of changes in trophoblast cell morphology and increases in cell proliferation cause the spherical conceptus to transform into an elongated and eventually a filamentous structure that occupies the majority of one uterine horn prior to implantation (Ashworth & Bazer 1989, Guillomot 1995, Robinson et al. 2006). The rapid expansion in trophectoderm greatly increases surface area contact with the uterine epithelium and increases the overall production of interferon-tau (IFNT), the maternal recognition of pregnancy factor in these species (Robinson et al. 2006, Ealy & Yang 2009). The timely achievement of both these events is required to maintain pregnancy in cattle and sheep (Thatcher et al. 2001, Inskeep & Dailey 2005).

Uterine gland secretions, also known as histotroph, are required for ovine conceptus elongation (Gray et al. 2001a, Brandao et al. 2004), and several uterine-derived cytokines and growth factors play critical roles in regulating pre- and peri-implantation conceptus development in cattle and sheep. Several members of the fibroblast growth factor (FGF) family are implicated in controlling peri-implantation development. At least, two of these factors, FGF2 and FGF10, are produced by the uterus and conceptus during peri-implantation development in cattle and sheep (Chen et al. 2000, Michael et al. 2006, Ocón-Grove et al. 2008, Cooke et al. 2009). Receptors for FGF2 and FGF10 and many other FGFs are evident in bovine and ovine blastocysts and peri-implantation bovine and ovine conceptuses (Chen et al. 2000, Ocón-Grove et al. 2008, Cooke et al. 2009, Munoz et al. 2009). One known activity of FGF2 and FGF10 in bovine trophoblast cell lines and blastocysts is the stimulation of IFNT mRNA and protein...
production (Michael et al. 2006, Ocon-Grove et al. 2008, Cooke et al. 2009, Rodina et al. 2009). Additional activities of these paracrine factors likely exist, and the following work was completed to determine if FGF2 and FGF10 mediate the migratory activity of ovine and bovine trophoblast cells. 

Trophoblast cell migration is one component to trophoblast cell reorganization and morphogenesis that likely plays an important role in mediating conceptus development during the peri-attachment period (Guillomot 1995, Ferretti et al. 2007, Bazer et al. 2009). Several uterine- and conceptus-derived factors induce trophoblast cell migration. These include epidermal growth factor (EGF), insulin-like growth factor 2 (IGF2), galectin-15 (SGAL15), WNT5A, and periostin (POSTN; Hayashi et al. 2007, Farmer et al. 2008, Kim et al. 2008, Ahn et al. 2009, Simmons et al. 2009, Dilly et al. 2010). Several signaling molecules have been linked with this activity, and several of the aforementioned factors utilize MAP kinases (MAPKs), phosphoinositide 3-kinase (PI3K), Rho-kinase, or a combination of these pathways to control migration rates (Hayashi et al. 2007, Kim et al. 2008, Dilly et al. 2010). The potential involvement of FGFs in trophoblast cell migration has not been examined in ruminant species, but specific FGFs regulate cell migration in other systems (Bottcher & Niehrs 2005, Tao et al. 2005, Natanson-Yaron et al. 2007, Kuriyama & Mayor 2009, Vergano-Vera et al. 2009). Also, an embryonic lethal phenotype exists in mice lacking FGF receptor 1 because of failures in extraembryonic and mesoderm cell migration during gastrulation (Ciruna et al. 1997, Rossant et al. 1997). A series of experiments were conducted to establish that FGF2 and FGF10 stimulate ovine and bovine trophoblast cell migration and determine whether specific MAPKs are required for this activity. The MAPKs examined in this study included MAPK3/1 (also known as ERK1/2), MAPK14 (also known as p38 MAPK), and MAPK8/9 (also known as SAPK/JNK).

Results

FGF2 and FGF10 stimulate migration of ovine trophoblast cells

Several FGFs, including FGF2 and FGF10, stimulate chemotaxis and migration in several cell types. Because these FGFs reside in the uterus during peri-implantation development in cattle and sheep, we set out to determine whether FGF2 and FGF10 regulate trophoblast cell migratory activity by examining how these factors impact oTr1 cell migration in vitro. This cell line was developed from an elongating ovine conceptus (day 15 of gestation; Farmer et al. 2008) and is used extensively to examine various aspects of trophoblast activity, including cell migration (Hayashi et al. 2007, Farmer et al. 2008, Ahn et al. 2009, Erikson et al. 2009, Simmons et al. 2009). Studies were completed using a previously described assay examining the movement of oTr1 cells through Corning Transwell inserts containing 8 μm pores (Farmer et al. 2008, Kim et al. 2008). Supplementation with 0.5 ng/ml FGF2 or FGF10 did not affect oTr1 cell migration but providing 5 ng/ml FGF2 or FGF10 increased (P<0.05) the percentage migrated cells after 8 h when compared with non-treated controls (Fig. 1A and B). Supplementing 50 ng/ml FGF2 did not induce further oTr1 migration rates, whereas 50 ng/ml FGF10 increased (P<0.05) migratory activity beyond what was observed in cells supplemented with 5 ng/ml FGF10.

FGF2 and FGF10 stimulate MAPK activity in oTr1 cells

Several MAPK pathways have been linked with trophoblast cell migration (Ferretti et al. 2007, Dilly et al. 2010). Because FGFs utilize MAPK signals for various purposes in many cell types (Turner & Grose 2010), studies were completed to identify specific MAPKs involved with basal- and FGF-induced migration of oTr1 cells. The first MAPKs examined were MAPK3/1. Western blot analyses (Fig. 2A) and subsequent analysis of band intensities (Fig. 2B) determined that both FGF2 and FGF10 increased (P<0.05) MAPK3/1 phosphorylation status within 5 min of treatment. This stimulation in phosphorylation remained greater (P<0.05) than controls for 60 min for MAPK1 and at least 120 min for MAPK3. Changes in phosphorylation status were not assessed after 120 min.

A separate set of studies determined that FGF2 and FGF10 also stimulated MAPK14 activation in oTr1 cells (Fig. 3). Increases (P<0.05) in MAPK14 phosphorylation

![Figure 1](image-url)
kinases were required for FGF mediation of oTr1 cell migration. Each inhibitor was added to cultures 2 h before FGF supplementation. Similar concentrations of each inhibitor were used in previous studies on oTr1 cells (Farmer et al. 2008, Kim et al. 2008). Western blot analyses verified that treating oTr1 cells with each inhibitor for 2 h prevented phosphorylation of each respective MAPK after FGF2 or FGF10 treatment (data not shown). None of the pharmacological inhibitors increased the incidence of apoptosis in oTr1 cells (data not shown).

In the absence of inhibitors, FGF2 and FGF10 increased (P<0.05) oTr1 cell migration (Fig. 5A and B). Pre-treatment with MAPK3/1, MAPK14, or MAPK8/9 prevented FGF2- and FGF10-induced increases in cell migration. The MAPK3/1 or MAPK14 appeared to act specifically by interfering with FGF2 and FGF10 activity because migratory activity in these groups were similar with cells not provided FGFs and with cells not receiving either pharmacological inhibitor (DMSO only). The MAPK8/9 inhibitor caused a nonsignificant decrease in oTr1 cell migration in non-FGF-treated cells (P=0.1 for both FGF2 and FGF10).

**FGF2 and FGF10 stimulate migration of bovine trophoblast cells**

A final study was completed to determine if FGF2 and FGF10 also influence the migratory activity of the CT1 bovine trophoblast cell line (Talbot et al. 2000, Michael et al. 2006). These cells migrated across 8 μm pores at a slower rate than oTr cells, and the assay time was extended to 12 h to compensate for this difference in basal migratory activity (data not shown). Supplementing the cultures with FGF2 or FGF10 also increased (P<0.05) oTr1 cell migration (Fig. 5A and B). Western blot analyses verified that treating oTr1 cells with each inhibitor were used in previous studies on oTr1 cells (Farmer et al. 2008, Kim et al. 2008). Western blot analyses verified that treating oTr1 cells with each inhibitor for 2 h prevented phosphorylation of each respective MAPK after FGF2 or FGF10 treatment (data not shown). None of the pharmacological inhibitors increased the incidence of apoptosis in oTr1 cells (data not shown).

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A samples (differences between pretreatment (time 0) and post-treatment are represented as mean fold differences (smaller band represents MAPK8. (B) The relative intensities of MAPK8/9. The larger molecular mass band represents MAPK9 and the studies). (A) Representative blots for oTr1 cell responses to FGF2 and (2011) Reproduction pregnancy in cattle and sheep (Oco´n-Grove et al. 2008). Both FGFs are produced in early pregnancy. peri-attachment conceptus development in ruminant studies into the biological activities of FGF2 and FGF10 during The overall aim of this study was to provide new clues CT1 cells with FGF2 or FGF10 influenced their migratory activity (Fig. 6). Migration of CT1 cells was increased (P<0.05) by supplementation with 0.5 ng/ml FGF2 and greater concentrations of FGF2 further increased cells migration (Fig. 6A). In FGF10-treated CT1 cells, supplementation at 0.5 ng/ml did not affect cell migration but supplementation with 5 or 50 ng/ml FGF10 increased (P<0.05) migration indices over non-treated controls (Fig. 6B).

Discussion

The overall aim of this study was to provide new clues into the biological activities of FGF2 and FGF10 during peri-attachment conceptus development in ruminant species. Both FGFs are produced in early pregnancy. FGF2 is produced primarily in luminal and glandular epithelium throughout the estrous cycle and early pregnancy in cattle and sheep (Ocón-Grove et al. 2008, Cooke et al. 2009). In the ewe, uterine luminal FGF2 protein concentrations increase at days 12–13 post-estrus coincident with conceptus elongation in this species (Ocón-Grove et al. 2008). It is unknown whether cattle also experience an increase in uterine FGF2 during conceptus elongation. FGF2 mRNA is also detected in bovine conceptuses throughout pre- and peri-implantation periods, but the predominant conceptus-derived FGF transcript identified in elongating and filamentous bovine conceptuses is FGF10 (Cooke et al. 2009).

The uterine stroma also produces FGF10 throughout the estrous cycle and early pregnancy and it is likely that some of this reaches the uterine lumen before implantation (Chen et al. 2000, Satterfield et al. 2008).

These FGFs influence several biological activities of trophoblast cells. One recently identified activity of FGFs in bovine trophoblast cells is the stimulation of IFNT production (Michael et al. 2006, Ocón-Grove et al. 2008, Cooke et al. 2009, Rodina et al. 2009). FGF2 and likely other FGFs increase IFNT mRNA and protein abundance in trophoblast cell lines and blastocysts by regulating the activity of a novel protein kinase C, termed PKC-δ (Yang et al. 2011). This project determined that FGF2 and FGF10 participate in other events associated with peri-implantation conceptus development in ruminants. The mechanisms controlling conceptus elongation are not well understood in ruminants, but there is ample evidence that uterine histotroph is required for elongation to proceed normally. In the ewe, conceptuses generated in ewes lacking uterine glands, the primary source of histotroph, fail to elongate properly (Gray et al. 2001b, 2002). Also, ovine conceptuses collected at day 12 post-mating failed to elongate after their removal from the uterus, but reacquired the ability to elongate after their transfer back into uteri of surrogates (Flechon et al. 1986).

In cattle, blastocysts cultured for extended periods in agarose tubes with medium containing large amounts of serum acquires an elongated-like phenotype, but the rate of this elongation is much slower than that observed.
FGF2/10 stimulation of trophoblast cell migration

FGF2 and FGF10 promote bovine trophoblast cell (CT1) migration. Cells were seeded onto Transwell inserts containing 8 μm pores (50,000 cells/insert) in serum-free medium containing 0, 0.5, 5, or 50 ng/ml FGF2 (A) or FGF10 (B). Cells migrating to the lower chamber of the insert were counted 12 h later. Results represent means and S.E.M. of fold differences relative to control values (n= 5 replicate studies in panel A and 4 replicate studies in panel B). Different superscripts denote differences (P<0.05) between FGF treatments within each panel.

Figure 6

This study demonstrated that supplementation with FGF2 or FGF10 increased ovine and bovine trophoblast cell migration in vitro. The oTr1 cell has been used previously to discover several uterine-derived migratory factors (Farmer et al. 2008, Dilly et al. 2010). Several other uterine factors are implicated in controlling conceptus development. The two uterine factors identified in the sheep are insulin-like growth factor-binding protein 1 (IGFBP1) and LGALS15. The expression of both factors increases in luminal and glandular epithelium around the time of conceptus elongation and filamentation, and supplementing these factors increased oTr1 cell migration and adhesion (Lewis et al. 2007, Farmer et al. 2008, Simmons et al. 2009). In cattle, IGFBP1 expression also increases around the time of elongation (Simmons et al. 2009), suggesting that it plays similar roles during peri-implantation development in both species. The same may not be said for LGALS15 because the bovine homolog is not produced in the bovine uterus during diestrus and early pregnancy (Lewis et al. 2007). It is unclear whether a paralog for LGALS15 contains a uterine-dependent expression profile or whether this factor or a related molecule is not required in bovids.

Another interesting facet of previous work is that these migratory factors have little influence of trophoblast proliferation. IGFBP1 and LGALS15 have little to no effect on oTr1 cell proliferation (Lewis et al. 2007, Farmer et al. 2008, Simmons et al. 2009). FGF2 and FGF10 also have little to no effect on bovine trophoblast or blastomere proliferation (Michael et al. 2006, Cooke et al. 2009, Rodina et al. 2009). These observations suggest that there is a divergence in the control of migratory and proliferative activities in trophoblast cells.

The MAPK signaling modules were examined to describe those utilized by FGF2 and FGF10 in regulating oTr1 cell migration. FGFs are well-known activators of MAPK pathways in several cells (Turner & Grose 2010). Examining the Ras/MEK/ERK pathway was of special interest given the importance of this pathway, and MAPK1, in particular, in promoting trophoblast lineage specification and placental cell differentiation in mouse embryos (Lu et al. 2008, Nadeau et al. 2009). FGF2 and FGF10 increased MAPK3/1 phosphorylation status in oTr1 cells. The MAPKs exhibited differential responses to FGF activation, with MAPK3 phosphorylation lasting longer than MAPK1 (>120 vs 60 min). The importance of this outcome remains unclear. Exposure to the MEK1/2 inhibitor prevented FGF2 and FGF10 from activating MAPK3/1, and exposure to this inhibitor prevented both FGFs from stimulating oTr migration. This implicates MAPK3 or MAPK1, or perhaps both, as mediators of FGF2- and FGF10-dependent migration in these cells. Other factors also utilize the Ras/MEK/ERK pathway to control ovine and bovine trophoblast migration. Kim et al. (2008) determined that IGF2 activated MAPK3/1 in oTr1 cells and stimulated oTr1 cell migration. EGF also activated MAPK3/1 in F3 cells, a bovine trophoblast line derived from a mid-gestation bovine placenta, and inhibiting these kinases interfered with EGF-induced cell migration and motility (Dilly et al. 2010, Hambruch et al. 2010).

The MAPK14 and MAPK8/9 (i.e. p38 and SAPK/JNK) pathways are also activated by FGF2 and FGF10 in oTr1 cells, and inhibiting these kinases prevented FGF-mediated migration in these cells. Several uterine factors regulate phosphorylation status of these MAPKs in oTr1 cells (Hayashi et al. 2007, Farmer et al. 2008, Kim et al. 2008), and these observations indicate that several MAPK systems must be activated to promote trophoblast migration. Additional signaling pathways not examined in this study, including the PI3K, FRAP1, and Rho-kinase systems, are also associated with uterine-dependent activation of trophoblast cell migration (Hayashi et al. 2007, Farmer et al. 2008, Kim et al. 2008, Dilly et al. 2010). The need for such multiplicity of signaling for controlling migratory activity in trophoblast cells is not clear. Perhaps, this reflects that a complex coordination of signaling modules is required during conceptus...
development and that several uterine factors may be needed to induce the correct combination of signaling events for conceptus elongation and peri-attachment development to proceed normally.

Although our understanding of conceptus development and elongation in ruminants is far from complete, this study describes a new activity for uterine- and conceptus-derived FGFs that may be of importance in regulating conceptus development during the second and third weeks of pregnancy in cattle and sheep. Insight also was made describing cellular mechanisms that respond to FGFs and control trophoblast cell migration.

Materials and Methods

Reagents

Unless indicated otherwise, cell culture reagents were purchased from Invitrogen Corp. Bovine recombinant (rb) FGF2 was purchased from R&D Systems (Minneapolis, MN, USA) and human recombinant (rh) FGF10 was purchased from Invitrogen Corp. Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Transwell inserts were purchased from Corning, Inc. (Lowell, MA, USA). Prolong Antifade reagent and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Corp. Pharmacological inhibitors for MAPKs (PD98059, SB203580, and JNK inhibitor I (cell permeable)) were purchased from EMD Chemicals (Gibbstown, NJ, USA). The proteinase and phosphatase inhibitors cocktails and antibodies recognizing phosphorylated or total MAPK3/1, MAPK14, MAPK8/9, and HRP-labeled secondary antibody (anti-rabbit) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The BCA protein Assay and BioMax film were purchased from ThermoFisher Scientific (Pittsburgh, PA, USA). PVDF membrane (Immobilon-P) was purchased from Millipore Co. (Bedford, MA, USA). ECL western blot detection system was purchased from GE Healthcare (Piscataway, NJ, USA). All other reagents were purchased from Sigma–Aldrich.

Trophoblast cell cultures

The ovine trophoblast cell line (oTr1) was developed by Dr Robert Burghardt (Farmer et al. 2008) and provided by Dr Thomas E Spencer (Texas A&M University). Cells were cultured on plastic (non-Matrigel coated) in DMEM/F12 medium containing 10% (v/v) Fetal bovine serum (FBS), 700 mM insulin, 100 mM non-essential amino acids (NEAA), and antibiotic–antimycotic (100 μg/ml penicillin G, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B). Cells were passaged by enzymatic dissociation (0.25% (w/v) trypsin) as described previously (Farmer et al. 2008). Cells were serum-starved by replacing medium with DMEM/F12 lacking FBS but containing all other supplements.

Bovine CT1 cells were propagated on Matrigel-coated plates in DMEM (with high glucose) containing 10% FBS, 100 μg NEAA, 55 μg β-mercaptoethanol, and antibiotic–antimycotic (100 μg/ml penicillin G, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B) at 38.5 °C with 5% CO2 in air as described previously (Kim et al. 2008) with minor modifications. Cells were serum-starved for 24 h before harvesting from plates. Cells (30,000 oTr1 cells or 50,000 CT1 cells in 100 μl serum-free medium) were seeded onto Transwell inserts (8 μm pores; Costar #3422). Treatments were added to each well (0, 0.5, 5, or 50 ng/ml FGF2 or FGF10; n = 3 wells/treatment). After 8 h for oTr1 cells and 12 h for CT1 cells, medium in the top chamber was removed and cells remaining on the top chamber were removed with a cotton swab. Cells migrating through to the bottom side of the insert evaluated by fixation in 4% (w/v) paraformaldehyde for 15 min at room temperature and staining with 8 μM DAPI. After staining, membranes were removed from inserts and mounted on a glass slides with the migrating surface facing up, overlaid with Prolong antifade and then with a coverslip. Migrated cells were counted in seven nonoverlapping locations in each membrane using a Nikon TE2000 inverted phase epifluorescence microscope and CoolPix CCD camera and NIS-Elements Software (Nikon Corp., Melville, NY, USA). Each study was repeated on at least three separate occasions with different batches of cells.

For studies examining the effects of pharmacological inhibitors on basal and FGF-induced cell migration, oTr cells were serum-starved for 24 h and seeded onto Transwell inserts. Cells were treated with 50 μM PD08059, 25 μM SB203580, 2 μM JNK inhibitor I, or vehicle only (<0.01% DMSO). After 2h, medium containing the inhibitors was replaced with FGF2- or FGF10-enriched medium (50 ng/ml). The migration assay was terminated after 8 h as described previously.

Western blot analyses

Cells (oTr1) were serum-starved for 24 h. Cells were harvested either immediately before adding FGFs (time 0) or 5, 15, 30, 60, or 120 min after treatment with 50 ng/ml FGF2 or FGF10. Cells were rinsed with Dulbecco’s PBS (DPBS) and dissolved in NP40 buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 20 mM EDTA, and 1% (v/v) NP40) supplemented with protease and phosphatase inhibitor cocktail. Cell lysates were sonicated and supernatant protein concentrations were determined using a BCA Protein Assay. Protein samples (20 μg) were loaded and separated on 10% (w/v) SDS-PAGE gels and transferred onto 0.45 μm PVDF. Membranes were blocked with 5% (w/v) non-fat dry milk in TBST (50 mM Tris–HCl pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween-20), then incubated overnight at 4 °C with antibodies against phosphor-specific MAPKs. HRP-conjugated anti-rabbit IgG was used in combination with ECL to visualize reactive bands after exposure to BioMax film. After

Migration assay

Trophoblast cell migration assays were completed as described previously (Kim et al. 2008) with minor modifications. Cells were serum-starved for 24 h before harvesting from plates. Cells (30,000 oTr1 cells or 50,000 CT1 cells in 100 μl serum-free medium) were seeded onto Transwell inserts (8 μm pores; Costar #3422). Treatments were added to each well (0, 0.5, 5, or 50 ng/ml FGF2 or FGF10; n = 3 wells/treatment). After 8 h for oTr1 cells and 12 h for CT1 cells, medium in the top chamber was removed and cells remaining on the top chamber were removed with a cotton swab. Cells migrating through to the bottom side of the insert evaluated by fixation in 4% (w/v) paraformaldehyde for 15 min at room temperature and staining with 8 μM DAPI. After staining, membranes were removed from inserts and mounted on a glass slides with the migrating surface facing up, overlaid with Prolong antifade and then with a coverslip. Migrated cells were counted in seven nonoverlapping locations in each membrane using a Nikon TE2000 inverted phase epifluorescence microscope and CoolPix CCD camera and NIS-Elements Software (Nikon Corp., Melville, NY, USA). Each study was repeated on at least three separate occasions with different batches of cells.

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detection, membranes were washed with TBST, stripped according to manufacturer instructions, and used to detect total MAPK protein. Multiple western blots generated from different batches of oTr cells were completed (n=3–4 for each MAPK), and band intensities were quantified after scanning using ImageJ Software (NIH, Bethesda, MD, USA). Representative immunoblots were photographed and presented in figures.

**Statistical analyses**

All analyses were performed by least squares ANOVA using the general linear model of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). Differences between individual means were compared using pairwise comparisons (PDIF) (probability of difference) analysis in SAS). Results were presented as the arithmetic mean ± S.E.M.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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