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Epidermal growth factor-stimulated extravillous cytotrophoblast motility is mediated by the activation of PI3-K, Akt and both p38 and p42/44 mitogen-activated protein kinases

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BACKGROUND: Trophoblast invasion is a temporally and spatially regulated scheme of events that can dictate pregnancy outcome. Evidence suggests that the potent mitogen epidermal growth factor (EGF) regulates cytotrophoblast (CTB) differentiation and invasion during early pregnancy. METHODS AND RESULTS: In the present study, the first trimester extravillous CTB cell line SGHPL-4 was used to investigate the signalling pathways involved in the motile component of EGF-mediated CTB migration/invasion. EGF induced the phosphorylation of the phosphatidylinositol 3-kinase (PI3-K)-dependent proteins, Akt and GSK-3β as well as both p42/44 MAPK and p38 mitogen-activated protein kinases (MAPK). EGF-stimulated motility was significantly reduced following the inhibition of PI3-K ($P < 0.001$), Akt ($P < 0.01$) and both p42/44 MAPK ($P < 0.001$) and p38 MAPKs ($P < 0.001$) but not the inhibition of GSK-3β. Further analysis indicated that the p38 MAPK inhibitor SB 203580 inhibited EGF-stimulated phosphorylation of Akt on serine 473, which may be responsible for the effect SB 203580 has on CTB motility. Although Akt activation leads to GSK-3β phosphorylation and the subsequent expression of β-catenin, activation of this pathway by 1-azakenpaullone was insufficient to stimulate the motile phenotype. CONCLUSION: We demonstrate a role for PI3-K, p42/44 MAPK and p38 MAPK in the stimulation of CTB cell motility by EGF, however activation of β-catenin alone was insufficient to stimulate cell motility.

Keywords: epidermal growth factor; extravillous cytotrophoblast; motility; PI3-K; p38 mitogen-activated protein kinases

Introduction

Cytotrophoblasts (CTBs) play a pivotal role in the development and maintenance of a successful pregnancy. Extravillous CTBs invade the underlying decidua, surround and migrate into the wall of the uterine spiral arteries through mechanisms that involve trophoblast-mediated apoptosis of endothelial and smooth muscle cells of the vessel wall (Ashton *et al*., 2005; Harris *et al*., 2006). Remodelling of the maternal uterine arteries transforms them from small diameter, high resistance vessels into large diameter, low resistance vessels (Robertson *et al*., 1973). The resulting increase in maternal blood flow is required to meet the increased demands of the developing feto-placental unit. Failure of the extravillous trophoblasts to adequately invade the uterine wall results in poor vessel remodelling and a reduction in placental perfusion, characteristic of common pregnancy complications such as pre-eclampsia, intrauterine growth restriction and pre-term labour (Brosens *et al*., 1972). Conversely, uncontrolled invasion is indicative of choriocarcinoma.

Human CTB invasion is controlled both temporally and spatially and involves the coordination of a number of distinct processes, including the breakdown of extracellular components through the synthesis and release of matrix metalloproteinases (MMPs), as well as migration towards the maternal spiral arteries. An important component of the migratory processes is cellular motility. Motility can be considered as a cycle of events starting with the extension of cellular processes, frontal adhesion, transcellular contraction and finally rear extension release. Orchestration of these complex events can be mediated through integrin/matrix interactions and/or

stimulation of growth factor receptor kinases by a number of external signals (Wells *et al*., 2002). We have previously reported that CTB motility is stimulated by hepatocyte growth factor (HGF) (Cartwright *et al*., 1999, 2002; Tse *et al*., 2002). More recently, we have established a role for epidermal growth factor (EGF) in regulating CTB migration (Barber *et al*., 2005; LaMarca *et al*., 2005, 2006).

EGF is a multifunctional growth factor that regulates a variety of fundamental cell properties, such as growth, differentiation, invasion and apoptosis in numerous cell types. Ligand binding induces receptor homo- or heterodimerization that is essential for the phosphorylation of multiple tyrosine (Tyr) residues and the activation of Tyr kinases. These Tyr residues provide docking sites for specific Src homology 2-containing proteins, such as phospholipase Cγ (Di Fiore *et al*., 1990; Olayioye *et al*., 2000; Yarden, 2001; Yarden *et al*., 2001). These interactions launch a variety of intracellular pathways that can involve mitogen-activated protein kinases (MAPKs) or the activation of phosphatidylinositol 3-kinase (PI3-K) (Olayioye *et al*., 2000; Yarden, 2001; Yarden and Sliwkowski, 2001). Phosphoinositides generated by PI3-K activity trigger activation of Akt kinases through direct binding to the pleckstrin homology domain and the subsequent phosphorylation of Akt at two conserved residues, serine^{473} (Ser^{473}) and threonine^{308} (Thr^{308}).

The aim of this study was to examine in more detail the EGF-mediated signalling events responsible for the motile component involved in CTB migration and invasion.

Materials and Methods

Cells and reagents

The human extravillous CTB cell line SGHPL-4 was derived from first trimester chorionic villous tissues. These cells are well characterized and share many characteristics with isolated primary cells, including the expression of cytokeratin-7, HLA class I antigen, HLA-G, BC-1 and CD9 (Cartwright *et al*., 1999; Cartwright and Balarajah, 2005). Cells were cultured in Ham's F10 Nutrient Mix (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 mg/ml) and 2 mM L-glutamine at 37°C in 5% CO_2. Recombinant human EGF was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). PD 98059, LY 294002, SB 203580, Akt inhibitor IV and 1-azakenpaullone were obtained from EMD Biosciences (Madison, WI, USA). The TCF β-catenin reporter plasmid, TOPFLASH (Kim *et al*., 2000) was obtained from Europa Bioproducts Ltd, Ely, UK.

Boyden chamber chemotaxis assay

A modified Boyden chamber assay was performed as previously described to examine cell migration (Lamarca *et al*., 2006). Briefly, 10 ng/ml of EGF diluted in serum-free medium was added to the lower compartment of the Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD, USA), and SGHPL-4 cells were serum-starved in medium containing 0.5% FBS for 48 h. The cells were then pre-treated with dimethylsulphoxide (DMSO) (vehicle), 10 μM LY 294002 (specific inhibitor to PI3-K) or 50 μM PD 98059 (specific inhibitor to p42/44 MAPK) for 30 min, trypsinized and subsequently added to the upper compartment of the Boyden chamber at a density of 5.6×10^4cells/well. After a 6 h migration period at 37°C, the cells on the upper side of the membrane were wiped off and the migrated cells were visualized by staining the cells that extended across the porous membrane (Diff-Quik Stain Set, Dade Behring, Inc., Newark, DE, USA). The stained membranes were visualized using a Nikon TE300 inverted epifluorescent microscope (DP Controller v1.2.1.108, Olympus Optical Company, LTD; Nikon USA, Lewisville, TX) and migration was quantified by counting the nuclei that passed through the filter. Stained nuclei from six fields of view (100×) for each experimental condition were counted and the data were expressed as the average number of migrated cells.

Invasion assay

Cellular invasion of SGHPL-4 cells in response to various inhibitors was assessed using the quantitative FluoroBlok invasion assay (BD Discovery Labware, Bedford, MA, USA) according to the manufacturer's instructions. SGHPL-4 cells were serum-starved for 24 h, washed with 1× phosphate-buffered saline (PBS) and pre-treated with DMSO (vehicle), 10 μM LY 294002 or 50 μM PD 98059 for 30 min at 37°C. The cells were trypsinized, resuspended in serum-free medium and seeded onto Matrigel-coated 8 μm FluoroBlok porous membrane inserts at a density of 2.5×10^5 cells per insert. The inserts were lowered into individual wells of a 24-well plate that contained 10 ng/ml of EGF and the plates were incubated at 37°C for 20 h to allow for cell invasion. Then, the invading cells were fluorescently labelled with Calcein AM (Molecular Probes, Eugene, OR, USA) as described previously (Lamarca *et al*., 2006), and relative fluorescence units were obtained with a fluorescent microtiter plate reader (FLUOstar optima, BMG Labtech, Durham, NC) from four replicates per experimental condition.

Western blotting

SGHPL-4 cells were serum-starved in 0.5% FBS/HamF10 for 24 h at 37°C. Cells were then treated in the absence of serum with 10 ng/ml of EGF or vehicle control (PBS) for 0, 5, 15, 30 or 60 min. Where indicated, cells were pre-treated with DMSO or the appropriate inhibitor for 30 min at 37°C prior to the addition of EGF. Cells were harvested in 1× RIPA (radioimmunoprecipitation assay) buffer containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and 1 mM phenylmethylsulphonyl fluoride (Sigma, St Louis, MO, USA), lysates were sheared 3× with a 27G1/2 needle and cell debris was removed by centrifugation at 18 000 g at 4°C. Total protein concentrations were estimated with the Micro BCA™ Protein Assay Kit (Pierce, Milwaukee, WI, USA) and an equal amount of total protein in each well was resolved on 8–10% sodium dodecyl sulphate (SDS)–polyacrylamide gels before transfer to polyvinylidene diflouride membranes (Amersham Biosciences, Piscataway, NJ, USA). Non-specific reactivity was blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Blots were incubated overnight with the following antibodies: rabbit anti-p42/44-Thr^{202}/Thr^{204}, rabbit anti-p42-total, rabbit anti-p38-Thr^{180}/Tyr^{182}, rabbit anti-phospho-Akt-Ser^{473} or rabbit anti-GSK-3β-Ser^9 (1:1000; Cell Signalling Technology, Beverly, MA, USA). The blots were then washed in TBST and incubated with goat or donkey anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Sigma or Amersham Biosciences, respectively) for 1 h at room temperature, and antigen–antibody complexes were detected using an enhanced chemiluminescence system (Amersham Biosciences). Blots were subsequently stripped in buffer containing 62.5 mM Tris pH 6.7, 2% SDS and 100 mM β-mercaptoethanol and tested for immunoreactivity to a rabbit polyclonal antibody to human β-actin (Sigma). Where indicated, western blots were scanned and the integrated intensity of each band determined using Scion Image for Windows version Beta 4.02. Results were expressed as a ratio to β-actin in the same sample.

Motility assays

Time-lapse digital image microscopy using an Olympus IX70 inverted microscope equipped with a Hamamatsu C4742-95 digital camera was performed to examine cell motility (Cartwright *et al*., 1999). The microscope and stage were enclosed within a heated (37°C) humidified atmosphere of 5% CO_2 (Solent Scientific, UK). SGHPL-4 cells were incubated in medium containing 0.5% FBS for 24 h, at which time they were transferred to the microscope chamber in fresh medium containing vehicle (PBS) or 10 ng/ml of EGF. Where appropriate, cells were pre-treated with the indicated inhibitors for 30 min at 37°C or the vehicle (DMSO) and subsequently transferred to the microscope chamber in the presence or absence of EGF. In accordance with previous studies, 10 ng/ml was found to be the optimal concentration of EGF to stimulate motility (data not shown) and was therefore used for the remainder of the study. Images were captured every 15 min over a period of 6 h and motility was quantified using Image Pro-Plus software (Media Cybernetics, USA). The distance moved was determined by randomly analysing at least 60 cells per treatment, and the experiment was performed a total of three times. Visualizing the cells by this method ensured that only viable cells were analysed throughout the 6 h period.

Transfections and luciferase assays

Transient transfections of SGHPL-4 cells with the β-catenin reporter plasmid TOPFLASH were performed in 6-well plates in the presence of 5% FBS using poly-L-ornithine (15 000 MW) from Sigma mixed with DNA at a ratio of 0.9:1 (w/w). Cells were incubated with the poly-L-ornithine/DNA mixture at a DNA concentration of 2.5 μg/ml at 37°C for 5 h. DMSO (30% in RPMI media) was added for 1 min, washed twice with PBS and fresh media (Hams F10, 10% FBS) was added. Analysis of the cells was carried out 24 h post-transfection. Luciferase assays were performed according to the manufacturer's instructions (BD Bioscience).

Statistical analyses

Data are presented as the mean + SEM. Data from treated groups were compared with vehicle control groups and significant differences were determined by one-way analysis of variance followed by Tukey's *post hoc t*-test (GraphPad Prism© Home, San Diego, CA, USA).

Results

PI3-k and p42/44 activation are required for EGF-mediated chemotaxis and invasion

We have previously reported that EGF induces *in vitro* CTB migration and invasion in a dose-dependent fashion (Barber *et al*., 2005; Lamarca *et al*., 2006). EGF was a potent chemotactic factor when used at 10 ng/ml in the Boyden chamber, and likewise stimulated the invasion of CTBs through an extracellular matrix. In the present study, these assays were used to investigate the regulatory mechanisms involving EGF and CTB invasiveness. EGF-stimulated SGHPL-4 cell migration and invasion (Fig. 1) was significantly inhibited by pre-treatment with specific inhibitors to p42/44 MAPK (PD 98059) or PI3-K (LY 294002), as compared with vehicle-treated cells. Chemotaxis was inhibited >5-fold in PD 98059- and LY 294002-treated cells when measured in the Boyden chamber ($P < 0.001$; Fig. 1a). Similarly, invasion through Matrigel was also significantly inhibited ($P < 0.001$) in SGHPL-4 cells pre-treated with these inhibitors as compared with vehicle-treated cells (Fig. 1b). These data implicate the involvement of both p42/44 MAPK and PI3-K signalling in EGF-regulated CTB chemotaxis and invasion and establish the validity of the model for subsequent studies of the motile mechanisms involved.

EGF stimulates CTB motility

An important component of CTB migration and invasion is cellular motility. We therefore determined whether PI3-K and or the MAPK pathways were involved in this aspect of the invasive process. We have previously reported that HGF stimulates not only invasion, but CTB motility through a mechanism involving PI3-K and MAPK activation (Cartwright *et al*., 1999, 2002). To determine whether EGF shared these characteristics with HGF, time-lapse microscopy was used to examine the motility of SGHPL-4 cells in both the presence and the absence of EGF.

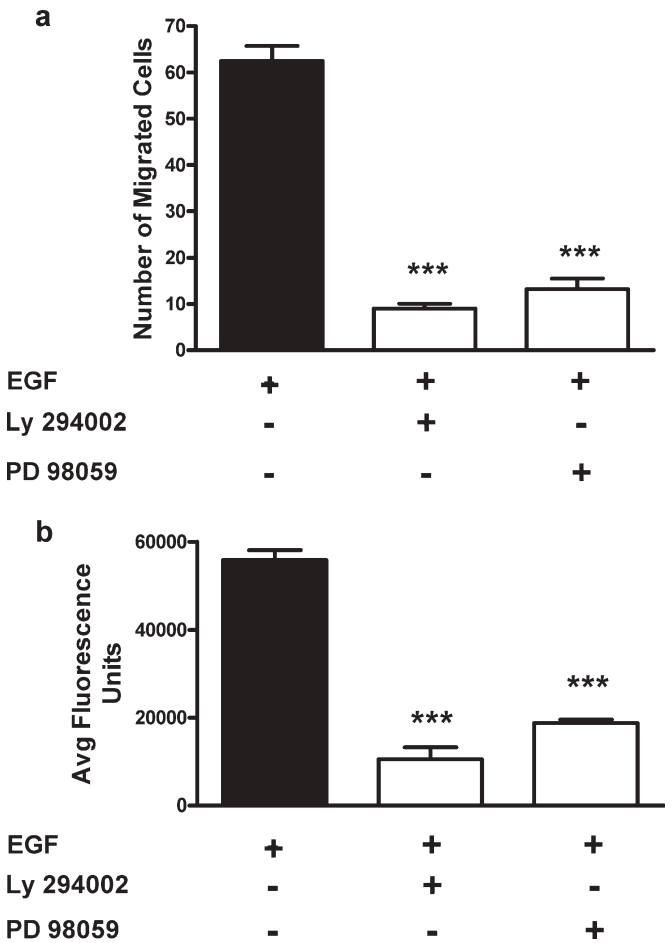

Figure 1: Involvement of mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3-K) signalling in cytotrophoblast (CTB) chemotaxis and invasion.
SGHPL-4 cells were pre-treated with 10 μM LY 294002-(specific inhibitor to PI3-K) or 50 μM PD 98059 (specific inhibitor to p42/44 MAPK) for 30 min, and cell migration and invasion assays were performed. Treatment with either LY 294002 or PD 98059 significantly inhibited epidermal growth factor (EGF)-induced (**a**) chemotaxis in the Boyden chamber and (**b**) invasion through Matrigel (***$P < 0.001$). The results presented are the mean + SEM from a representative experiment and each experiment was performed a total of four times.

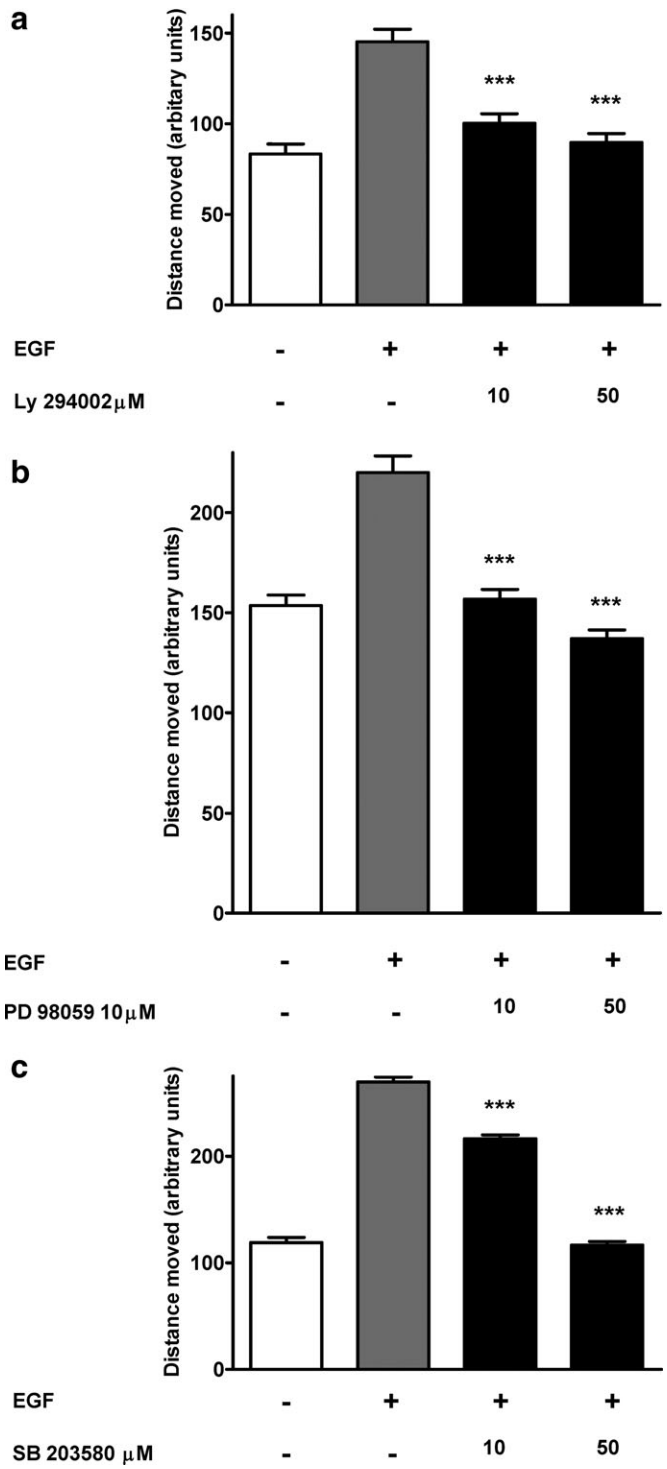

Figure 2: Regulation of CTB motility by PI3-K, p42/44 MAPK and p38 MAPK signalling pathways.
Time-lapse digital microscopy was performed on cells that were pre-treated for 30 min with the indicated doses of either vehicle (dimethylsulphoxide, DMSO) or (**a**) LY 294002, (**b**) PD 98059 or (**c**) SB 203580 (p38 MAPK inhibitor). Cells were then stimulated with 10 ng/ml EGF and motility was monitored over a 6 h period. The data presented in each panel are the mean distance moved + SEM from three independent experiments. The effect of each inhibitor was assessed independently and therefore was compared with contemporaneous control and EGF wells (***$P < 0.001$).

To investigate whether either PI3-K or p42/44 MAPK were involved in SGHPL-4 motility, time-lapse microscopy was performed after pre-treatment with specific inhibitors to these pathways. Inhibition of either PI3-K or p42/44 MAPK significantly reduced the motility of SGHPL-4 cells in a dose-dependent manner (Fig. 2a and b). In addition, treatment with SB 203580, a specific inhibitor of p38 MAPK, inhibited CTB motility in a dose-dependent manner with statistical significance achieved at 10 μM, the lowest dose tested (Fig. 2c).

EGF induces the phosphorylation of MAPKs and PI3-K-regulated proteins

Our functional studies established a role for the PI3-K, p38 and p42/44 MAPK pathways in the regulation of EGF-stimulated cell motility. In order to dissect these pathways and identify possible points of interaction, we studied the time course of activation for each pathway. In these studies, phosphorylation of both p42/44 MAPK and downstream targets of PI3-K, Akt and GSK-3β were activated within 5 min of stimulation (Fig. 3). Although phosphorylation of Akt (Ser^{473}) and p38

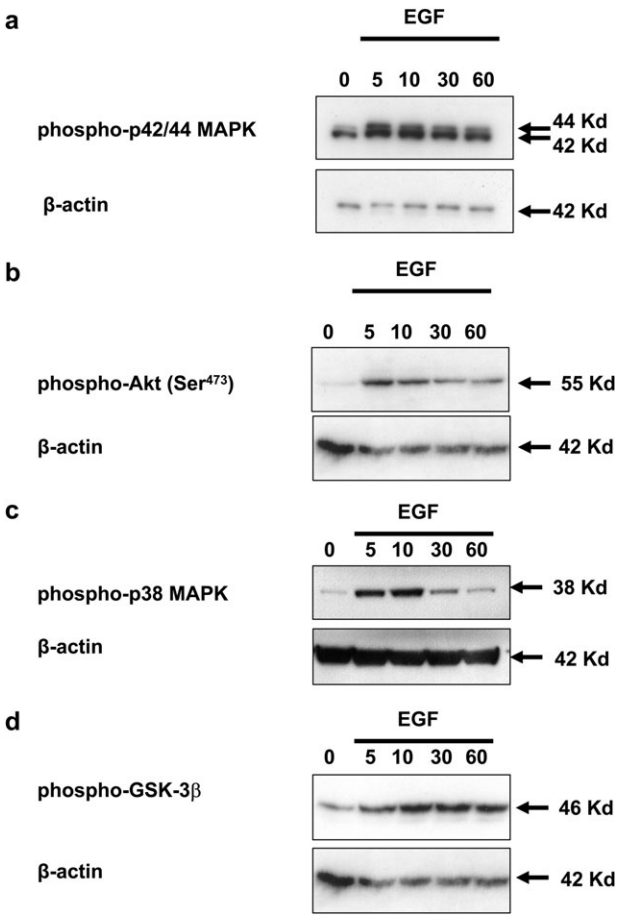

Figure 3: PI3-K-mediated p42/44 MAPK activation.
SGHPL-4 cells treated with 10 ng/ml of EGF or vehicle (PBS) for 0, 5, 15, 30 or 60 min. Cell lysates were collected and equal amounts of total protein were subjected to western-blot analyses for (**a**) phospho-p42/44, (**b**) phospho-Akt, (**c**) phospho-p38 MAPK, (**d**) phospho-GSK-3β. Human β-actin was used as an internal loading control. The results presented are representative of at least three independent experiments.

(Thr^{180}/Tyr^{182}) MAPK remained greater than the control for up to 60 min, there was a rapid decline after ~10 min of stimulation (Fig. 3a and b). In contrast, the phosphorylation state of both p42/44 MAPK and GSK-3β remained high throughout the experimental period (Fig. 3c and d).

Akt involvement in EGF-stimulated trophoblast motility

To further explore the role of the PI3-K/Akt pathway in EGF-mediated cell motility, a specific Akt inhibitor was used. Pre-treatment with Akt inhibitor IV resulted in dose-dependent inhibition of trophoblast motility (Fig. 4). Notably, significant trophoblast motility remained when treated with higher concentrations of the Akt inhibitor. However, increasing the concentration of Akt inhibitor IV beyond 2.5 μM had a significant effect on CTB viability.

Inhibition of GSK-3β does not affect EGF-stimulated trophoblast motility

Activation of Akt has been implicated in the activation of a number of cellular targets including GSK-3β, a protein kinase that phosphorylates a number of downstream targets including the multifunctional transcription factor β-catenin (Frame *et al*., 2001). In the active state (unphosphorylated), GSK-3β phosphorylates β-catenin, which targets this transcription factor for proteasomal degradation (Frame and Cohen, 2001). In the inactive phosphorylated form, GSK-3β is unable to phosphorylate β-catenin, which allows for the stabilization of β-catenin and the transcriptional activation of downstream target genes (Frame and Cohen, 2001). Our results demonstrate that GSK-3β was rapidly phosphorylated, and therefore inactivated, following treatment with EGF (Fig. 3d). Furthermore, Fig. 5 shows that GSK-3β phosphorylation was inhibited by the PI3-K inhibitor LY 294002.

To establish whether phosphorylation of GSK-3β resulted in β-catenin activation, we used the luciferase reporter construct containing the β-catenin/Tcf binding site (TOPFLASH). Stimulation of SGHPL-4 cells with EGF led to a 1.75-fold increase in reporter activity (Fig. 6a). As a result of inhibiting basal GSK-3β activity using 1-azakenpaullone, there was a 2-fold increase in reporter activity at least equivalent to that obtained with 10 ng/ml EGF. Inhibition of Akt using Akt inhibitor IV resulted in a significant dose-dependent inhibition of reporter activity at concentrations previously shown to significantly inhibit trophoblast motility. However, activation of β-catenin alone using 1-azakenpaullone at different concentrations was unable to stimulate trophoblast cell motility (Fig. 6b).

EGF activates p42/44 MAPK in a PI3-K-independent fashion

Our studies indicate that MAPK (both p42/44 and p38) and PI3-K (Akt) signalling are important regulators of CTB motility. To address whether the MAPKs and PI3-K pathways interact with each other, western-blot analysis was performed for phospho-Akt (Ser^{473}) (Fig. 7a and b) and phospho-p42/44 MAPK (Fig. 7c). Inhibition of PI3-K with LY 294002 inhibited the phosphorylation of Akt (Ser^{473}), whereas the p42/44 MAPK inhibitor PD 98059 had no effect (Fig. 7b and c). Interestingly inhibition of p38 MAPK by SB 203580 had a significant inhibitory effect on Akt activation and the phosphorylation of ser^{473} in the absence of EGF. The addition of DMSO had no effect on either basal or EGF-stimulated phosphorylation of either Akt or p42/44 MAPK.

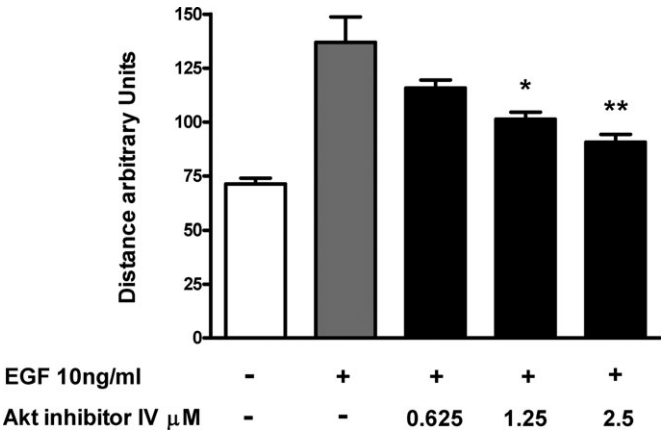

Figure 4: Regulation of CTB motility by Akt.
Time-lapse digital microscopy was performed on cells pre-treated with the indicated doses of either (**a**) Akt inhibitor IV or vehicle (DMSO) for 30 min and then stimulated with 10 ng/ml of EGF or PBS. Motility was monitored for 6 h. Data are presented as the mean + SEM from three independent experiments. Data from treated groups were compared with vehicle control groups and significant differences were determined by one-way analysis of variance followed by Tukey's *post hoc t*-test (*$P < 0.05$, **$P < 0.01$).

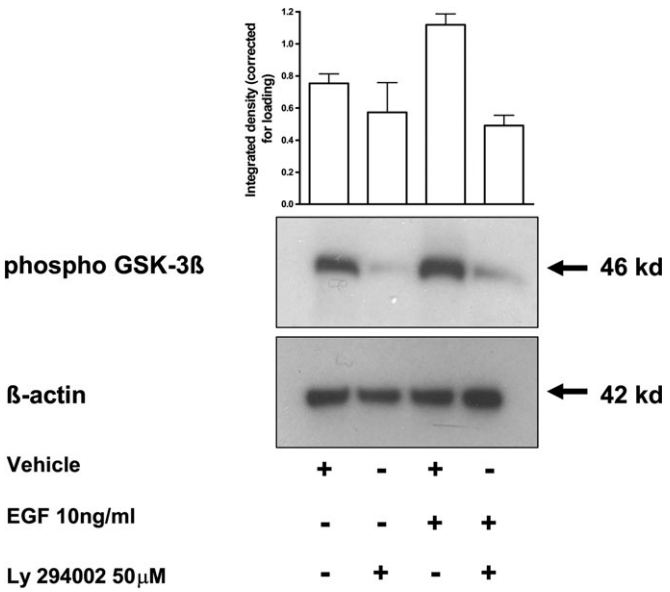

Figure 5: EGF-mediated GSK-3β.
SGHPL-4 cells were pre-treated with 50 μM LY 294002 for 30 min. Cells were subsequently treated with 10 ng/ml of EGF or vehicle (PBS) for 30 min. Cell lysates were collected and equal amounts of total protein per well were subjected to western-blot analyses for phospho-GSK-3β. Human β-actin was used as an internal control to verify equal sample loading. The top panel is the integrated density of the individual bands corrected using actin and is the mean + SEM of three separate experiments. The lower panel shows a representative blot of both GSK-3β and actin.

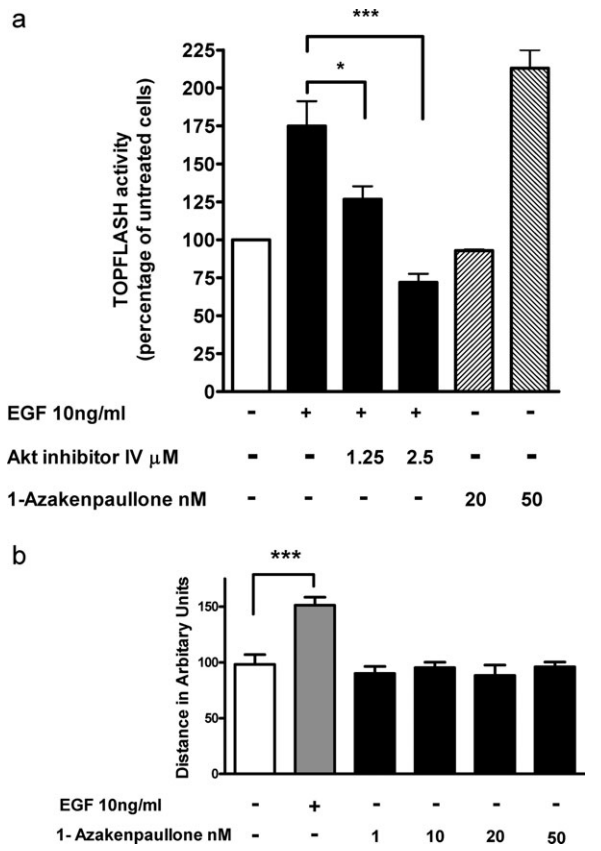

Figure 6: EGF-mediated β-catenin activation.
(**a**) SGHPL-4 cells were transiently transfected with the β-catenin reporter plasmid TOPFLASH. Twenty-four hours post-transfection, cells were treated with EGF (6 h) in the presence or absence of the Akt inhibitor IV (1.25 and 2.5 μM). Independent activation of β-catenin was demonstrated using the GSK-3β inhibitor 1-azakenpaullone (20 and 50 nM). (**b**) The effect of β-catenin activation on SGHPL-4 cell motility was determined following inhibition of GSK-3β with 1-azakenpaullone. Values shown are the mean + SEM of triplicate experiments $*P < 0.05$, $***P < 0.001$.

Basal phosphorylation of p42/44 MAPK was unaffected by inhibition with PD 98059; however, inhibition of PI3-K with LY 294002 resulted in a 50% reduction in the phosphorylation of Akt. This result is consistent with our previous finding that LY 294002 inhibits basal trophoblast motility (Cartwright *et al*., 2002). A similar but smaller effect on Akt phosphorylation was also observed following the inhibition of p38 MAPK.

Discussion

Invasion is a complex biological process involving the coordinated regulation of extracellular matrix remodelling through the secretion and activation of MMPs and stimulation of cellular motility. Although there have been studies examining the signal transduction mechanisms involved in EGF-stimulated trophoblast migration and invasion, very little is known about the motile component of these processes. Using time-lapse microscopy, pharmacological inhibitors and western-blot analysis, we have identified two independent pathways responsible for the regulation of trophoblast motility by EGF. The first results in the activation of p42/44 MAPK; the second is mediated through the phosphorylation of Akt and is independently activated by both PI3-K and more interestingly p38 MAPK.

EGF stimulates the migration and invasion of first and second trimester human CTBs as well as the secretion of MMP-2 and -9 (Bass *et al*., 1994; Staun-Ram *et al*., 2004; Anteby *et al*., 2004). In agreement with these studies and those of Qiu *et al*. (2004), who used the transformed trophoblast cell line HTR8neo, we were able to demonstrate EGF-stimulated migration and invasion using the first trimester extravillous CTB cell line SGHPL-4. Examination of the pathways involved using the PI3-K inhibitor LY 294002 and the p42/44 MAPK inhibitor PD 98059 corroborated the findings in previous studies (Qiu *et al*., 2004).

Having established a role for EGF in the regulation of migration and invasion and thereby validating our model, we proceeded to examine these processes in more detail by identifying post-receptor events responsible for the motile component of CTB invasiveness. Using time-lapse microscopy, we assessed cell movement over time. As we previously demonstrated, EGF induced CTB motility (Barber *et al*., 2005), and using molecular and pharmacological probes, we demonstrate a role for PI3-K, p42/44 MAPK and p38 MAPK in this process. Subsequent analysis indicated PI3-K dependent phosphorylation of Akt (Ser^{473}), and the involvement of Akt in EGF-regulated motility was confirmed using Akt inhibitor IV. To further explore the consequences of Akt activation, we investigated the involvement of the downstream target GSK-3β. GSK-3β is a multi-functional enzyme involved in the regulation of glycogen and protein synthesis and is known to be regulated by EGF in fibroblasts and MCF-7 cells. It is a well-established component of the Wnt signalling pathway and is also thought to play an important role in many other cellular processes, such as proliferation, differentiation and motility (Frame and Cohen, 2001). Most recently, it has been shown to regulate HGF-mediated trophoblast cell survival and invasive differentiation in trophoblasts (Dash *et al*., 2005; Pollheimer *et al*., 2006). Unlike most protein kinases, GSK-3β activity is inhibited following phosphorylation and consequently, a number of transcription factors are activated including β-catenin (Frame and Cohen, 2001). β-catenin is a multi-functional protein that is important for cell adhesion, but also mediates cytoplasmic effects leading to transcriptional activation. In the absence of specific activating signals, β-catenin is located either at adherin junctions or bound in a high molecular weight multi-protein complex that includes GSK-3β. In the absence of external stimuli, β-catenin is phosphorylated by GSK-3β, and this retains the β-catenin within the cytoplasm where it undergoes ubiquitination and subsequent degradation (Kimelman and Xu, 2006). Inhibition of GSK-3β kinase activity by its phosphorylation prevents the phosphorylation of β-catenin that is then able to translocate to the nucleus and associate with transcription factors of the Lef/Tcf family to initiate gene expression (Frame and Cohen, 2001).

In the present study, we demonstrate that in trophoblasts EGF phosphorylates GSK-3β, and using a luciferase reporter construct containing the β-catenin/Tcf binding site

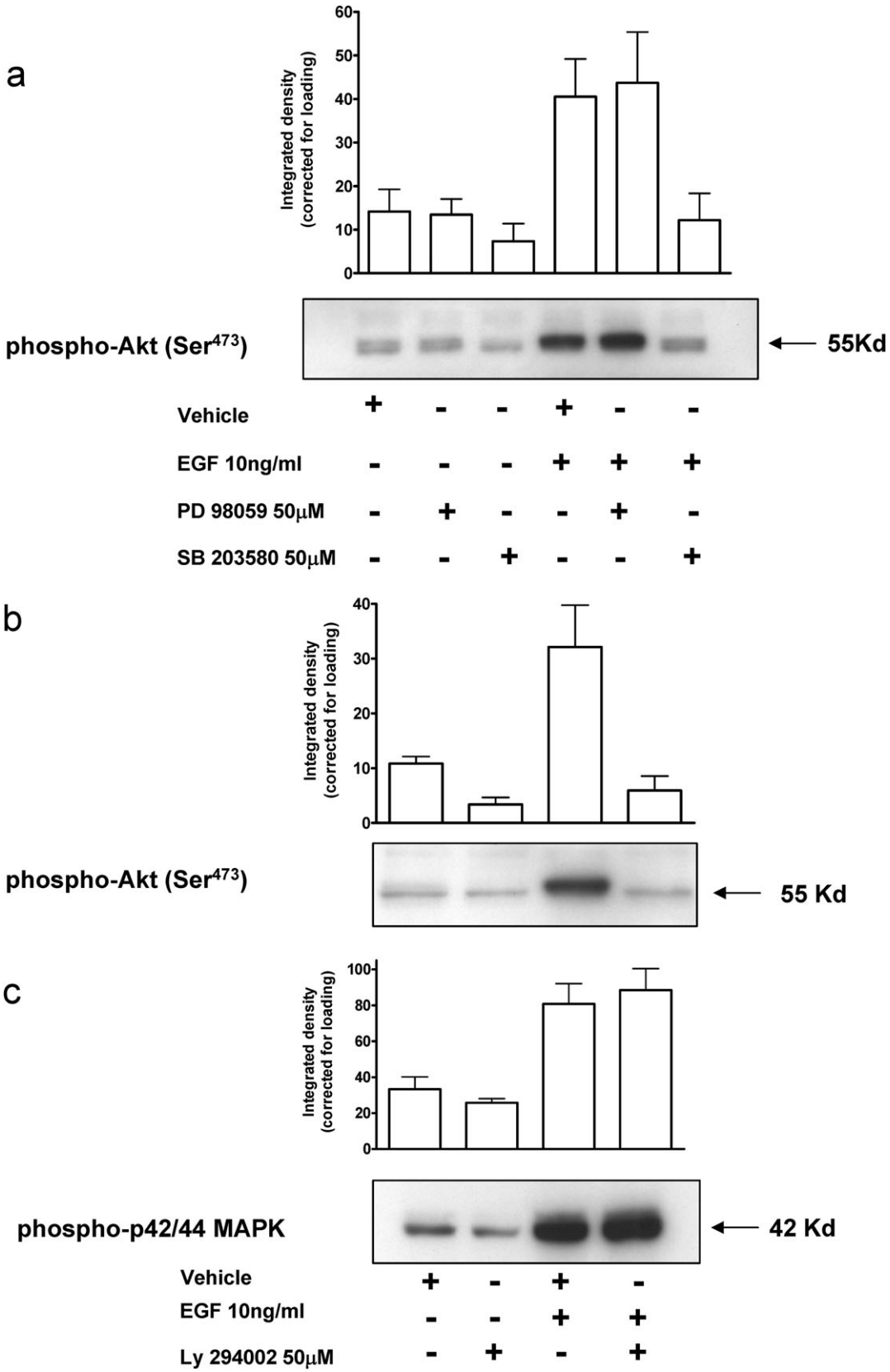

Figure 7: Phosphorylation of MAPKs and PI3-K-regulated proteins by EGF.
SGHPL-4 cells were treated with either the appropriate inhibitor or the vehicle control (DMSO) for 60 min. Cells were then stimulated with 10 ng/ml of EGF for 30 min in the presence or absence of (**a**) 50 μM PD 98059 or SB 203580 (**b** and **c**) 50 μmM LY 2984002. Cell lysates were collected and equal amounts of total protein were subjected to western-blot analyses. The bar chart represents the integrated density of the individual bands corrected for actin and is the mean + SEM of three separate experiments. The figure shows a representative example for each inhibitor.

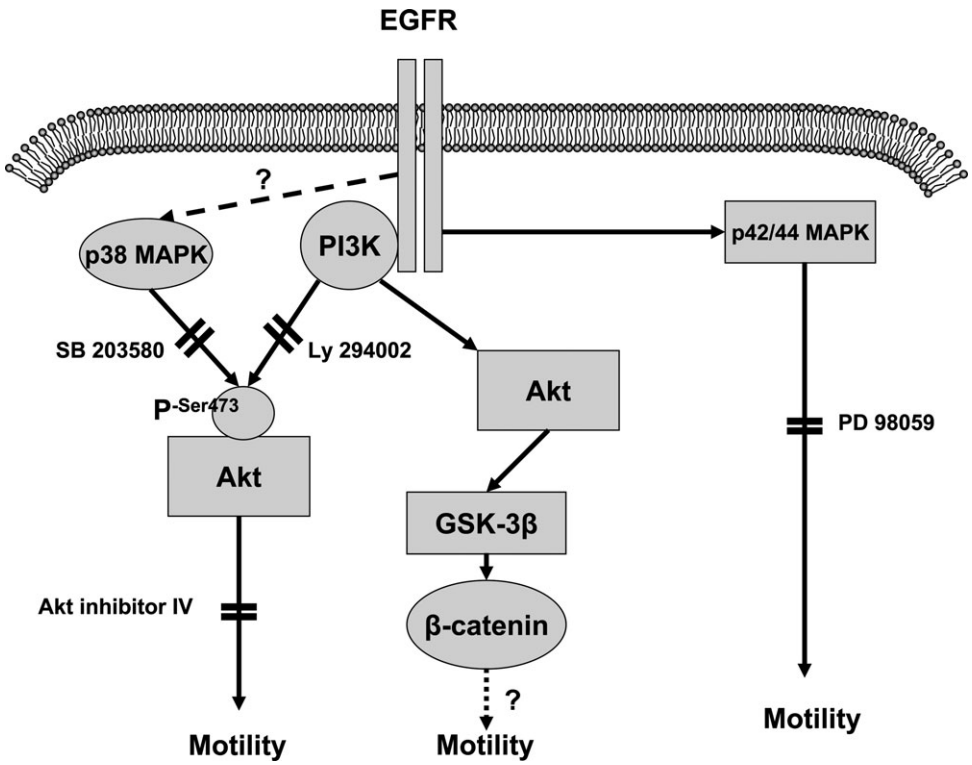

Figure 8: A schematic depicting the regulation of trophoblast cell motility by EGF.
Stimulation of CTB with EGF via the EGF receptor (EGFR) results in the activation of PI3-K and p42/44 MAPK. Inhibition of either pathway results in inhibition of EGF-stimulated motility. Akt is phosphorylated and therefore activated by EGF through the activation of PI3-K but not p42/44 MAPK. Inhibition of p38 MAPK inhibits both phosphorylation of Akt and CTB motility. Although EGF activates β-catenin, direct activation of β-catenin had no effect on motility.

(TOPFLASH), stimulates β-catenin gene expression. Further analysis showed that this process was mediated through the activation of the PI3-K/Akt pathway. However, when this pathway was activated independently of EGF receptor by inhibiting GSK-3β, there was no effect on trophoblast motility. These results would indicate that either inactivation of GSK-3β and the subsequent increase in gene transcription by β-catenin was not involved in the regulation of trophoblast cell motility or that activation alone is insufficient to stimulate motility. Whether parallel activation signalling pathways, such as the MAPKs, are required warrants further investigation.

Another known signalling molecule responsive to EGF is p38 MAPK. This protein kinase is a member of the MAPK family and has largely been associated with cellular stress responses and apoptosis. In the placenta, activation of p38 MAPK by EGF may play a role in the differentiation of third trimester CTBs (Johnstone *et al*., 2005). However, its involvement in cell migration and particularly trophoblast cell motility is not well established. In this study, we demonstrate that inhibition of p38 MAPK by SB 203580, the p38α and p38β isoform-specific MAPK inhibitor, had a significant inhibitory effect on trophoblast cell motility. These results were contrary to our previous findings involving HGF stimulation where similar doses had no significant effect. In vascular smooth muscle cells, p38 MAPK exists in a complex with Akt, and activation of p38 MAPK leads to recruitment of MAPKAPK-2 to the p38 MAPK–Akt complex, its phosphorylation, and thus activation. MAPKAPK-2 then phosphorylates Akt on Ser^{473}, leading to full Akt activation (Taniyama *et al*., 2004). A summary of our results are presented in Fig. 8.

In conclusion, EGF stimulates extravillous trophoblast cell motility. Following stimulation with EGF, there was an independent activation of PI3-K and p42/44 MAPK, and inhibition of both of these pathways had a significant inhibitory effect on trophoblast cell motility. Inhibition of PI3-K but not p42/44 MAPK led to inhibition of Akt phosphorylation. Interestingly, inhibition of p38 MAPK also resulted in the inhibition of EGF-induced trophoblast motility and Akt phosphorylation, indicating convergence of the PI3-K and p38 MAPK pathways in the control of trophoblast cell motility. As inhibition of β-catenin had no effect on EGF-stimulated CTB motility, the targets for Akt activation under these circumstances remain to be defined.

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