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Journal of Cell Science 123, 3413

doi:10.1242/jcs.080663

There were errors published in *J. Cell Sci.* **123**, 3189-3200.

In Fig. 4, the concentration of staurosporine (STS) was mistakenly given as 50 mM, rather than the correct concentration of 50 nM (lines 9 and 15 in the legend).

Moreover, the first sentence in the 'Signal transduction inhibitors' section in the Materials and Methods (page 3198) should read:

Rapamycin, staurosporine and wortmannin were purchased from Calbiochem (Merck Chemicals Ltd, Nottingham, UK) and reconstituted in DMSO (Sigma).

The authors apologise for these mistakes.

ERK5 is required for VEGF-mediated survival and tubular morphogenesis of primary human microvascular endothelial cells

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Accepted 5 June 2010
Journal of Cell Science 123, 3189-3200

doi:10.1242/jcs.072801

Summary

Extracellular signal-regulated kinase 5 (ERK5) is activated in response to environmental stress and growth factors. Gene ablation of *Erk5* in mice is embryonically lethal as a result of disruption of cardiovascular development and vascular integrity. We investigated vascular endothelial growth factor (VEGF)-mediated ERK5 activation in primary human dermal microvascular endothelial cells (HDMECs) undergoing proliferation on a gelatin matrix, and tubular morphogenesis within a collagen gel matrix. VEGF induced sustained ERK5 activation on both matrices. However, manipulation of ERK5 activity by siRNA-mediated gene silencing disrupted tubular morphogenesis without impacting proliferation. Overexpression of constitutively active MEK5 and ERK5 stimulated tubular morphogenesis in the absence of VEGF. Analysis of intracellular signalling revealed that ERK5 regulated AKT phosphorylation. On a collagen gel, ERK5 regulated VEGF-mediated phosphorylation of the pro-apoptotic protein BAD and increased expression of the anti-apoptotic protein BCL2, resulting in decreased caspase-3 activity and apoptosis suppression. Our findings suggest that ERK5 is required for AKT phosphorylation and cell survival and is crucial for endothelial cell differentiation in response to VEGF.

Key words: ERK5, VEGF, AKT, Endothelial, Angiogenesis, Signal transduction

Introduction

Angiogenesis defines the formation of new blood vessels from pre-existing vessels and is crucial for normal physiological vascular development, as well as for the pathological development of certain diseases, including diabetic retinopathy, atherosclerosis and cancer (Carmeliet, 2005). VEGF is a potent inducer of angiogenesis and is vital for vascular development, both during embryogenesis and in early neonatal life (Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999). Furthermore, VEGF is a key regulator of endothelial cell survival in the tumour vasculature (Benjamin and Keshet, 1997; Liu et al., 2000). It activates a number of receptor tyrosine kinases that are present on endothelial cells. VEGF receptor-1 (VEGFR-1/Flt-1) and VEGFR-2 (Flk-1/KDR) are present on vascular endothelial cells, whereas expression of VEGFR-3 (Flk-4) is confined to lymphatic endothelial cells (Cross et al., 2003; Lohela et al., 2009; Olsson et al., 2006). Numerous studies have revealed that activation of VEGFR-2 by the VEGF-A ligand is responsible for mediating the majority of physiological effects of VEGF in endothelial cells, including proliferation, migration, survival and permeability (Holmes et al., 2007). Identification of intracellular signalling pathways involved in VEGF–VEGFR-2 signalling has been subject to intense research in an attempt to decipher the crucial components downstream of VEGFR-2 (Holmes et al., 2007).

Mitogen-activated protein kinases (MAPKs) regulate the transduction of extracellular growth factor or stress-induced stimuli from the cell membrane to nuclear or cytoplasmic targets, and are crucial for the control of numerous cellular processes, including proliferation, migration and survival in response to these extracellular cues (Qi and Elion, 2005). Four major MAPK signalling cascades have been identified in mammalian cells: extracellular signal-regulated kinases 1 and 2 (ERK1/2), Jun-N-terminal kinases 1, 2 and 3 (JNK1–JNK3), p38 MAPKs (p38α, -β, -γ and -δ), and the most recently discovered MAPK, ERK5 (Chang and Karin, 2001; Roberts et al., 2009; Wang and Tournier, 2006). Each of these MAPKs is able to phosphorylate and activate a range of downstream substrates, resulting in specific intracellular responses (Lewis et al., 1998).

The ERK5 signalling module consists of MEKK2/3 (MAPK/ERK kinase kinase 2 and 3), which phosphorylates MEK5 (MAPK/ERK kinase 5), which in turn activates ERK5 by dual phosphorylation of Thr218 and Tyr220 within a T-E-Y activation motif. The physiological importance of the ERK5 signalling cascade was revealed in studies of targeted deletion of either *Erk5* (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003), *Mek5* (Wang et al., 2005), or *Mekk3* (Yang et al., 2000) in mice. Deletion of any of these genes resulted in death between embryonic day 9.5 (E9.5) and E11.5 as a result of hemorrhaging caused by severe abnormalities in cardiovascular development and compromised vascular integrity. Importantly, targeted deletion of *Erk5* in murine endothelial cells, but not in other cell types such as cardiomyocytes and hepatocytes, was also found to result in cardiovascular defects and disrupted vascular integrity, a phenotype that is identical to that of the global *Erk5* knockout mouse (Hayashi et al., 2004; Hayashi and Lee, 2004). These data suggest that although ERK5 is expressed in a variety of tissues, expression of ERK5 within the endothelium is crucial to preserve

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vascular integrity, and to maintain normal endothelial cell function (Roberts et al., 2009).

ERK5 is activated by a range of extracellular stimuli, including the pro-angiogenic growth factors VEGF and FGF-2 (fibroblast growth factor-2) in endothelial cells (Hayashi et al., 2004). The finding that ERK5 is required for tumour-associated angiogenesis (Hayashi et al., 2005) raises the possibility that ERK5 is a crucial component of the VEGF-signalling cascade in endothelial cells. However, the physiological importance of VEGF-stimulated ERK5 activation, and the downstream targets of VEGF-induced ERK5 activity have yet to be determined.

In this study, we addressed this issue by profiling VEGF-stimulated ERK5 activation in primary human dermal microvascular endothelial cells (HDMECs), and characterised the functional effects of modulation of ERK5 activation in models of distinct stages of the angiogenic process. Activation of ERK5 was observed under conditions of both VEGF-induced HDMEC proliferation and VEGF-induced tubular morphogenesis of HDMECs in a three-dimensional collagen gel. However, only VEGF-stimulated tubular morphogenesis was affected by ERK5 downregulation. We show that ERK5 activity is required for VEGF-mediated activation of AKT and the resulting phosphorylation of BAD, leading to the prevention of endothelial cell apoptosis. Together, our data reveal for the first time, that ERK5 is a crucial mediator of VEGF-induced endothelial cell survival during conditions of tubular morphogenesis. Furthermore, our results suggest that the ERK5 pathway represents a viable target for the development of specific inhibitors aimed at preventing tumour-associated angiogenesis.

Results

VEGF stimulates sustained ERK5 activity in human endothelial cells during proliferation and differentiation

We used primary HDMECs to study the role of VEGF-mediated ERK5 activity. In response to VEGF stimulation, HDMECs proliferate on a gelatin matrix, but undergo tubular morphogenesis to form capillary vessels in a 3D collagen gel (Fig. 1A). Cell cycle analysis following stimulation with VEGF for 24 hours revealed that cells within collagen gel structures were cell cycle arrested (Fig. 1B). By contrast, VEGF-treated HDMECs plated on a gelatin matrix underwent proliferation, demonstrated by a significant number of cells in the S and G2–M phases of the cell cycle (Fig. 1B). The proliferation and differentiation of endothelial cells represent distinct aspects of the angiogenic response to VEGF in vivo, where endothelial cells must first digest the basement membrane, migrate and proliferate to form a sprout, before eventually differentiating into a functional lumen-containing vessel (Koike et al., 2004; Montesano et al., 1983). Immunoblot analysis of cell lysates using a phospho-ERK5 antibody, which is directed against the phosphorylated Thr218 and Tyr220 residues within the T-E-Y motif in the ERK5 activation loop, revealed that VEGF stimulation induced a

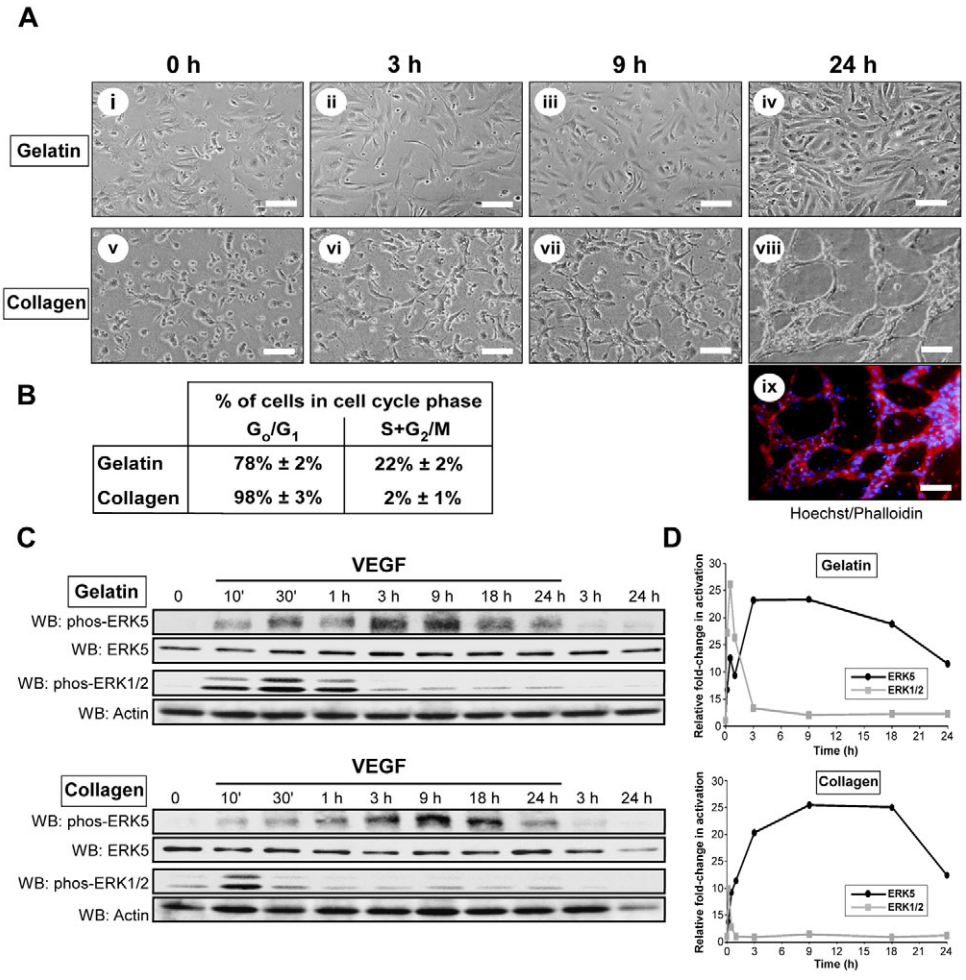

Fig. 1. VEGF stimulates activation of ERK5 under conditions of HDMEC proliferation and tubular morphogenesis.
(**A**) Morphology of HDMECs on gelatin and collagen I matrices. Serum-starved HDMECs were seeded on gelatin (i–iv), or between two layers of collagen I (v–viii). Unstimulated cells were fixed immediately (0 h), or at 3, 9 or 24 hours after stimulation with 50 ng/ml VEGF on both matrices. HDMECs plated on a collagen I matrix underwent VEGF-induced tubular morphogenesis by 24 hours (viii). Cells were fixed, HDMEC actin stress fibres, and nuclei were stained with Rhodamine-coupled Phalloidin (red) and Hoechst 33342 (blue), respectively (ix). Scale bars: 100 μm. (**B**) Analysis of cell cycle distribution of VEGF-stimulated HDMECs plated on gelatin and collagen I matrices. HDMECs were seeded on a gelatin matrix, or between two layers of collagen I before stimulation with 50 ng/ml VEGF for 24 hours. Cells were harvested and the percentage of cells in phases G_0–G_1 or S+(G_2–M) of the cell cycle were determined by FACS analysis. (**C**) HDMECs were serum starved for 20 hours before seeding on a gelatin matrix, or between two layers of collagen I, and stimulated with 50 ng/ml VEGF. Cells were lysed at the indicated times, and cell lysates were resolved by 10% SDS-PAGE, and analysed by western blotting (WB). (**D**) The degree of ERK5 and ERK1/2 phosphorylation, relative to actin, was quantified by densitometric analysis. Results are from one experiment, which is representative of three.

sustained activation of ERK5 in HDMECs on both gelatin and collagen matrices (Fig. 1C,D). VEGF did not induce any changes in the total level of the ERK5 protein (Fig. 1C) in HDMECs on either matrix. In contrast to the activation of ERK5, VEGF induced a relatively transient activation of ERK1/2, which returned to basal levels by 3 hours on a gelatin matrix and by 1 hour in a collagen gel (Fig. 1C,D).

VEGF-stimulated ERK5 activity is required for tubular morphogenesis but not cell proliferation

It is well established that VEGF is a potent inducer of endothelial cell proliferation in vitro (Ferrara and Henzel, 1989; Kroll and Waltenberger, 1997; Leung et al., 1989). To explore the potential involvement of ERK5 in HDMEC proliferation and tubular morphogenesis we used two separate siRNA duplexes targeting *ERK5*. Both of these duplexes downregulated ERK5 protein expression levels by 97%, as determined by western blot analysis (Fig. 2A). HDMECs plated on a gelatin matrix underwent a doubling in cell number following stimulation with VEGF for 3 days (Fig. 2B). *ERK5* silencing had no significant effect on VEGF-induced cellular proliferation (Fig. 2B). By contrast, siRNA-mediated *ERK5* silencing had a profound effect on VEGF-induced HDMEC tubular morphogenesis, because endothelial cells failed to form a network of vessels in collagen gel in response to VEGF

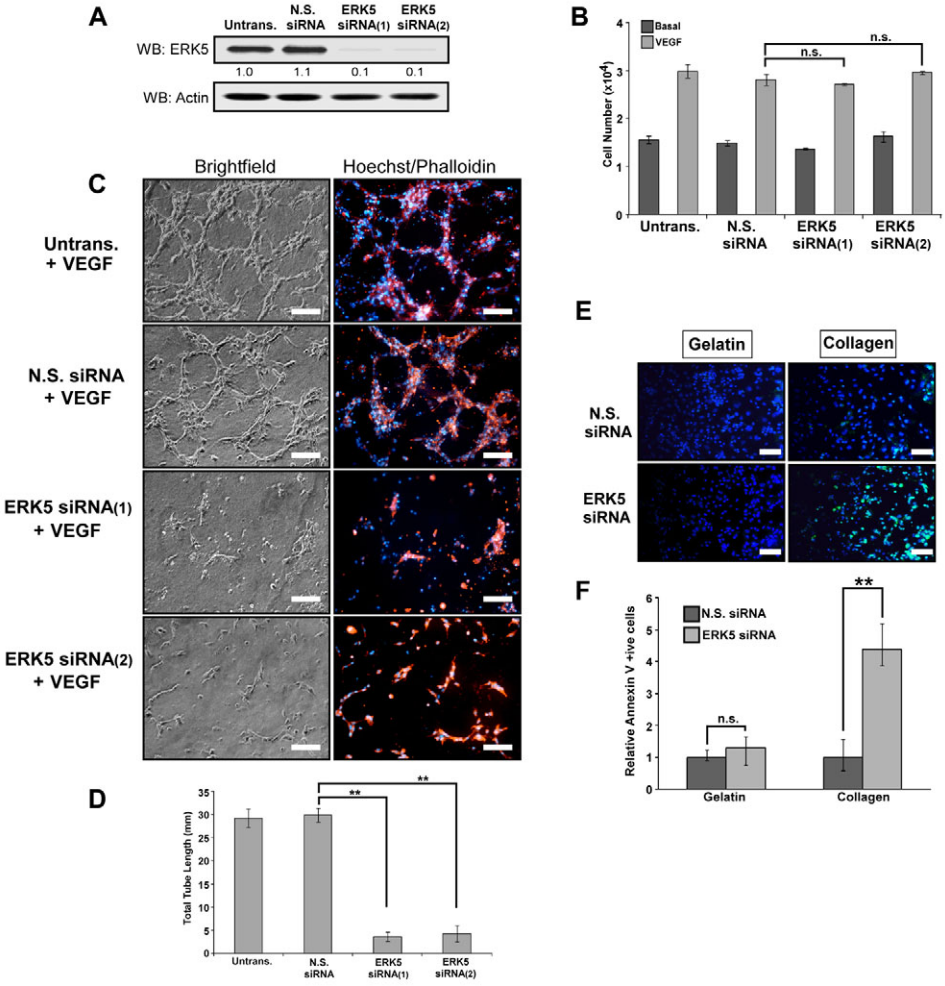

Fig. 2. Downregulation of ERK5 expression inhibits VEGF-induced HDMEC tubular morphogenesis but not proliferation. (**A**) HDMECs were transfected with 10 nM *ERK5* siRNA1, 10 nM *ERK5* siRNA2, 10 nM non-silencing (N.S.) siRNA or left untransfected (Untrans.). Cells were lysed 48 hours after transfection, and separated on a 10% SDS-PAGE gel before western blot (WB) analysis with antibodies directed against ERK5 and actin, as indicated. ERK5 expression, relative to actin, was quantified by densitometric analysis. (**B**) 24 hours after transfection, HDMECs were seeded at 1.5×10^4 cells per well on a gelatin matrix for 24 hours, before serum starvation for 20 hours, followed by stimulation with or without VEGF (50 ng/ml) for 72 hours. Cell numbers were quantified by Cell-Titer Glo® luminescence assay. Data are presented as cell number (n=3, mean ± s.d.; n.s., not significant). (**C**) 24 hours after transfection, HDMECs were serum starved for 20 hours and seeded between two layers of collagen I before stimulation with VEGF (50 ng/ml) for 24 hours. Cells were fixed, HDMEC actin stress fibres and nuclei were stained with Rhodamine-coupled Phalloidin (red) and Hoechst 33342 (blue), respectively. (**D**) The total length of tubular structures was quantified from three fields (×10 objective) per well (n=3, mean ± s.d.; $^{**}P<0.01$). (**E**) HDMECs were transfected with 10 nM *ERK5* siRNA (5 nM *ERK5* siRNA1 + 5 nM *ERK5* siRNA2) or with 10 nM of non-silencing (N.S.) siRNA. 24 hours after transfection, cells were serum starved for 20 hours, before seeding on gelatin or between two layers of collagen I, and stimulation with VEGF (50 ng/ml). 1 hour after VEGF stimulation, cells were stained with annexin V Alexa Fluor® 488 conjugate (green) and Hoechst 33342 (blue). (**F**) Quantification of early apoptosis in siRNA-transfected HDMECs after 1 hour of tube formation (collagen) or proliferation (gelatin) relative to N.S. siRNA control (n=3, mean ± s.d.; $^{**}P<0.01$; n.s., not significant).

(Fig. 2C,D). Cells appeared rounded, with pyknotic hypercondensed nuclei, indicative of cell death. Assessment of early apoptosis of HDMECs by annexin V staining of phosphatidylserine levels on the outer layer of the plasma membrane revealed that within collagen gels, cells treated with *ERK5* siRNA displayed a fourfold increase in apoptosis compared with cells transfected with non-silencing (N.S.) control siRNA (Fig. 2E,F). By contrast, *ERK5* siRNA-treated cells plated on a gelatin matrix showed no significant difference in the number of annexin V-positive cells, compared with that of the N.S. siRNA-treated cells (Fig. 2E,F). This data suggests that in HDMECs, ERK5 is required for VEGF-stimulated survival during tubular morphogenesis, but is not required during VEGF-induced cellular proliferation. In contrast to ERK5, siRNA-mediated silencing of *ERK1* and *ERK2* expression inhibited VEGF-mediated proliferation of HDMECs (supplementary material Fig. S1B). Silencing of *ERK1* had a small inhibitory effect on tubular morphogenesis, with tube length reduced by 15%, whereas silencing of *ERK2* had no effect on tubular morphogenesis (supplementary material Fig. S1C,D). These data reveal that, in HDMECs, ERK5 and ERK1/2 appear to have distinct roles; ERK5 regulating tubular morphogenesis and ERK1/2 regulating cellular proliferation.

ERK5 is required for VEGF-stimulated AKT phosphorylation and suppression of caspase-3 activity during tubular morphogenesis

The profound effect of silencing *ERK5* on HDMEC tubular morphogenesis with a concomitant increase in annexin V staining (Fig. 2E), led us to analyse the activation of pathways regulating apoptosis in HDMECs. VEGF stimulated phosphorylation of the VEGFR-2 on both gelatin and collagen matrices; an effect that was not altered by silencing *ERK5* expression (Fig. 3A). VEGF-induced activation of the serine/threonine kinase AKT/protein kinase B (PKB) is crucial for protecting endothelial cells against apoptosis (Fujio and Walsh, 1999; Gerber et al., 1998b). We analysed the phosphorylation status of AKT on Ser473 and Thr308 in HDMECs in response to acute VEGF stimulation when plated on a collagen matrix and a gelatin matrix. Treatment of HDMECs with siRNA duplexes against *ERK5* resulted in decreased phosphorylation of AKT by approximately 50% in response to VEGF on a gelatin matrix compared with untransfected cells and cells transfected with N.S. siRNA. By contrast, on a collagen matrix the inhibition was more profound, with AKT phosphorylation reduced by at least 94% (Fig. 3A). Importantly, the total level of AKT protein was not affected on either matrix, thus indicating that the inhibition of ERK5 activity was affecting AKT phosphorylation (Fig. 3A). AKT is known to regulate the activity of a number of signalling proteins, including the pro-apoptotic protein BAD (Datta et al., 1997) and the anti-apoptotic protein BCL2 (Pugazhenthi et al., 2000). Phosphorylation of BAD on Ser136 by AKT prevents BAD from interacting with BCL2 by sequestering BAD to the 14-3-3 protein (Datta et al., 1997; Zha et al., 1996). Acute VEGF-stimulation of HDMECs resulted in increased BAD phosphorylation on Ser136; an affect that was more pronounced on the collagen matrix (Fig. 3A). Treatment of HDMECs with siRNA duplexes against *ERK5* resulted in a decrease in VEGF-induced phosphorylation of BAD (Fig. 3A). Furthermore, silencing of *ERK5* also inhibited the ability of VEGF to stimulate increased *BCL2* mRNA expression in HDMECs: an effect that was more pronounced within a collagen gel (Fig. 3B).

Activation of the protease family of caspases represents one of the terminal stages of signal transduction pathways that lead to apoptosis in endothelial cells (Pober et al., 2009). We analysed the effect of silencing *ERK5* on the activity of caspase-3/7 in HDMECs plated on a gelatin or collagen matrix 1 hour, 6 hours and 24 hours after stimulation with VEGF, using a fluorescent-based assay. Comparison of the two matrices revealed that after 24 hours under basal conditions, caspase-3/7 activity was induced on the collagen gel; an effect that was suppressed by addition of VEGF (Fig. 4F).

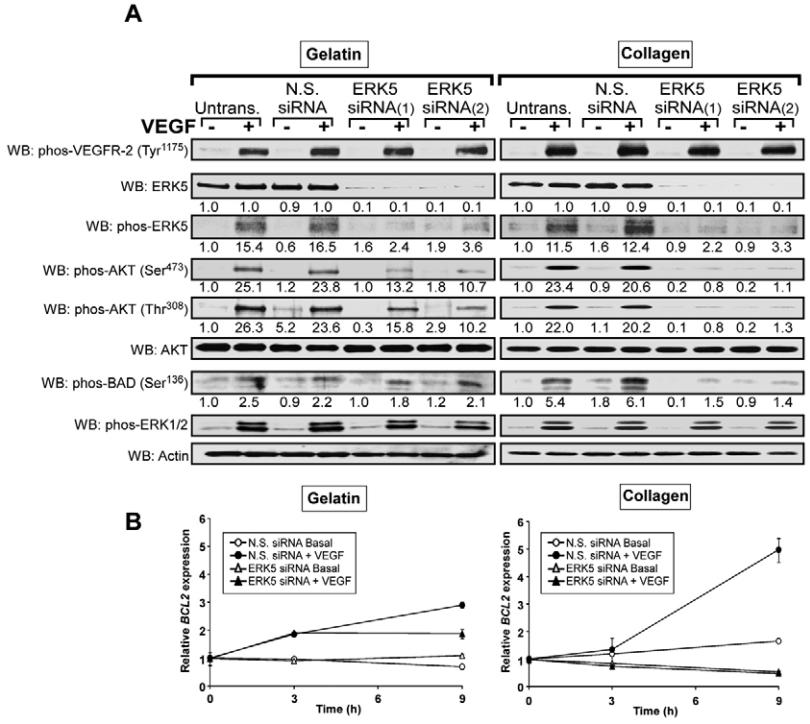

Fig. 3. ERK5 activation is required for VEGF-stimulated phosphorylation of AKT and BAD and maintaining BCL2 expression levels during tubular morphogenesis. (**A**) HDMECs were transfected with 10 nM *ERK5* siRNA1, 10 nM *ERK5* siRNA2, 10 nM non-silencing (N.S.) siRNA or left untransfected (Untrans.). 24 hours after transfection, cells were serum starved for 20 hours before plating on a gelatin matrix, or between two layers of a type I collagen gel and stimulated, or not, with VEGF (50 ng/ml) for 10 minutes. Cells were lysed in RIPA buffer and cell lysates were resolved by 10% SDS-PAGE and analysed by western blotting (WB). The expression of ERK5 relative to actin was quantified by densitometric analysis. (**B**) HDMECs were transfected with 5 nM *ERK5* siRNA1 and 5 nM *ERK5* siRNA2, or with 10 nM non-silencing (N.S.) siRNA. 24 hours after transfection, cells were serum starved for 20 hours, before plating on a gelatin matrix, or onto a collagen type I gel and stimulated with VEGF (50 ng/ml) for 3 and 9 hours. Total RNA was extracted and *BCL2* mRNA levels were quantified by qRT-PCR. Data are presented as level of *BCL2* mRNA relative to the value at 0 hour (n=3; mean ± s.d.).

Addition of staurosporine (STS), a known inducer of apoptosis in endothelial cells (Kabir et al., 2002), was used as a positive control and induced maximal caspase-3/7 activity on both matrices (Fig. 4A–F). Silencing of *ERK5* only induced caspase-3/7 activity in HDMECs plated in a collagen matrix; an effect that was evident 1 hour after cells had been plated in a collagen gel (Fig. 4B). Loss of ERK5 induced maximal apoptosis after 6 hours, and was still evident after 24 hours; an effect that was confined to the collagen matrix (Fig. 4C–F). The presence of VEGF was not sufficient to suppress ERK5-induced cleaved caspase-3 expression at any time point (Fig. 4A–F). To confirm the role of active caspase-3 in HDMEC apoptosis caused by loss of ERK5, we analysed the levels of the 17 kDa and 19 kDa cleaved caspase-3 proteolytic fragments by western blotting. Loss of ERK5, following siRNA-mediated downregulation, resulted in increased levels of cleaved caspase-3 only in HDMECs plated in a collagen gel, an effect that could not be reversed by addition of VEGF for 6 hours (Fig. 4G,H). Addition of STS was sufficient to induce the appearance of the 17 kDa and 19 kDa cleaved caspase-3 fragments on both gelatin and collagen matrices (Fig. 4G,H). Taken together, these data show that under conditions of tubular morphogenesis, ERK5 is crucial for mediating VEGF-stimulated AKT activation, and the resultant suppression of caspase-3 activity, facilitating endothelial cell survival. Conversely, on a gelatin matrix, where proliferation occurs, ERK5 is partly required for VEGF-mediated AKT activation, but is not required for suppression of caspase-3 activity or endothelial cell survival.

Constitutive activation of ERK5 in HDMECs leads to activation of AKT and suppression of caspase-3 activity

To confirm the role of ERK5 in the regulation of AKT activation and suppression of cleaved caspase-3 levels, we manipulated ERK5 activity by transiently transfecting HDMECs with plasmids expressing either Flag-tagged wild-type ERK5 [ERK5(wt)], or Flag-tagged dominant-negative ERK5 [DN-ERK5(AEF)], which cannot be phosphorylated by MEK5, together with hemagglutinin (HA)-tagged constitutively active MEK5 [CA-MEK5(D)]. In agreement with previous studies (Barros and Marshall, 2005; Schweppe et al., 2006), co-transfection of HDMECs with ERK5(wt) and CA-MEK5(D) induced an increase in ERK5 phosphorylation (Fig. 5). This increase in ERK5 phosphorylation was augmented by stimulation with VEGF, and blocked by co-transfection of DN-ERK5(AEF) and CA-MEK5(D) (Fig. 5). Co-transfection of ERK5(wt) and CA-MEK5(D) resulted in enhanced phosphorylation of AKT on both Ser473 and Thr308 in both the absence and the presence of VEGF (Fig. 5). Furthermore, enhanced phosphorylation of BAD on Ser136 and increased expression of BCL2 was also observed (Fig. 5). Analysis of cleaved caspase-3 levels revealed that expression of ERK5(wt) and CA-MEK5(D) resulted in suppression of cleaved caspase-3 levels even in the

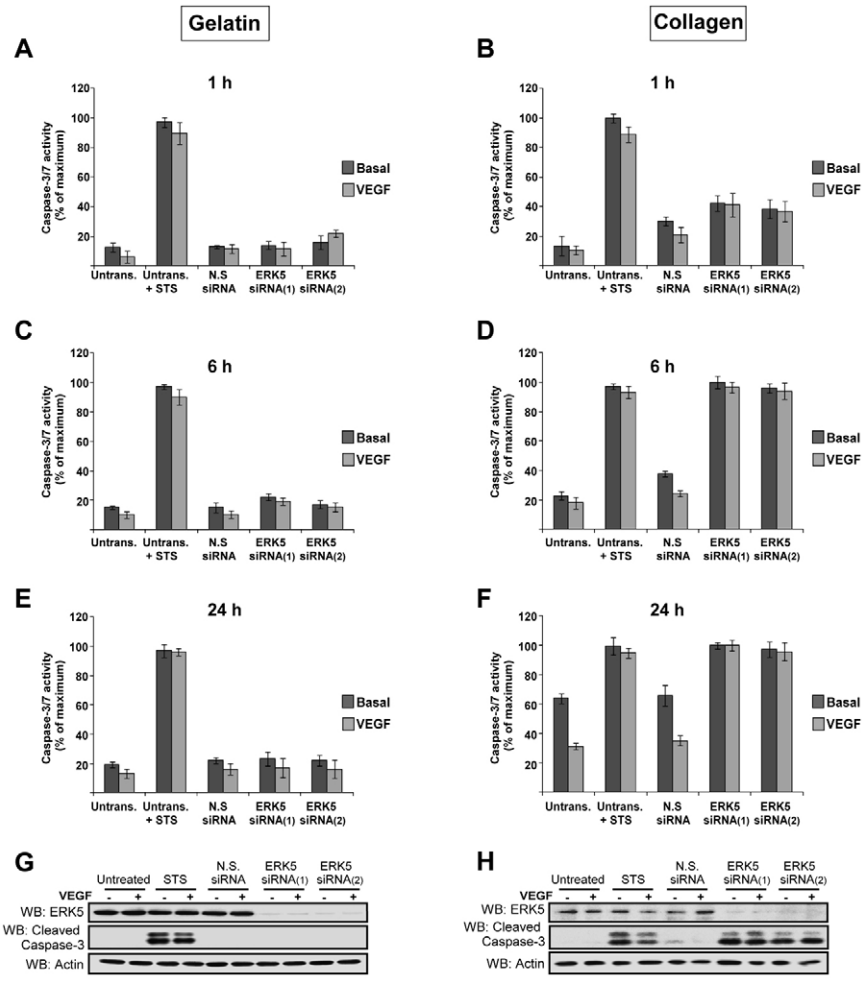

Fig. 4. Downregulation of ERK5 induces activation of caspase-3/7 during tubular morphogenesis. HDMECs were transfected with 10 nM *ERK5* siRNA1, 10 nM *ERK5* siRNA2, 10 nM non-silencing (N.S.) siRNA or left untransfected (Untrans.). 24 hours after transfection, cells were serum starved for 20 hours, before plating on a gelatin matrix, or between two layers of a collagen I gel. Cells were stimulated with VEGF (50 ng/ml) and/or 50 mM staurosporine (STS), as indicated. Cells were lysed in Caspase-Glo® 3/7 reagent at 1 hour (**A**,**B**), 6 hours (**C**,**D**) or 24 hours (**E**,**F**) after treatment with VEGF or STS. Data are presented as percentage of maximal caspase-3/7 activity (n=3; mean ± s.d.). (**G**,**H**) 6 hours after stimulation with VEGF, or following treatment with 50 mM staurosporine (STS), as indicated, cells were lysed in LDS buffer, and separated on a 4–12% gradient NuPAGE® gel, followed by western blot (WB) analysis with antibodies directed against ERK5, cleaved caspase-3 and actin, as indicated.

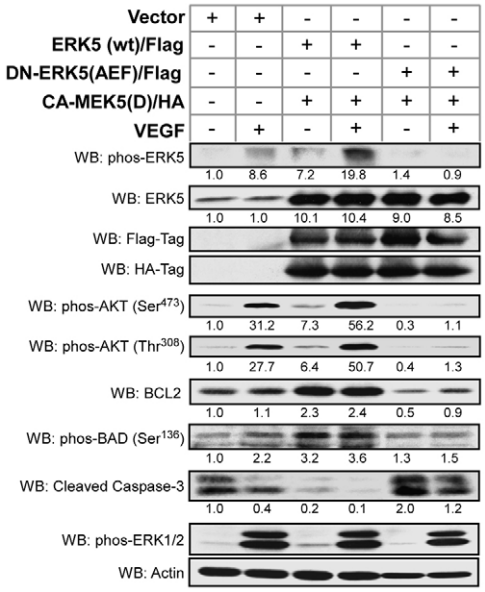

Fig. 5. Co-transfection of ERK5 and constitutively active MEK5(D) stimulates phosphorylation of ERK5 and AKT and induces expression of BCL2 whilst suppressing caspase-3 activity. HDMECs were transiently transfected with 2.5 μg ERK5(wt) and 2.5 μg CA-MEK5(D), with 2.5 μg DN-ERK5(AEF) + 2.5 μg CA-MEK5(D) or mock-transfected with 5.0 μg empty pcDNA3.1 (Vector). 24 hours after transfection, HDMECs were serum-starved for 20 hours before plating between two layers of collagen I and stimulation with VEGF (50 ng/ml) for 24 hours. Cells were stimulated again with VEGF (50 ng/ml) for 10 minutes before lysis in LDS sample buffer. Proteins were separated by SDS-PAGE followed by western blot (WB) analysis. The degree of phosphorylation of ERK5, AKT Ser473, AKT Thr308, BAD Ser136 and the expression levels of ERK5 and BCL2 relative to actin was quantified by densitometric analysis. Results are from one experiment, which is representative of three.

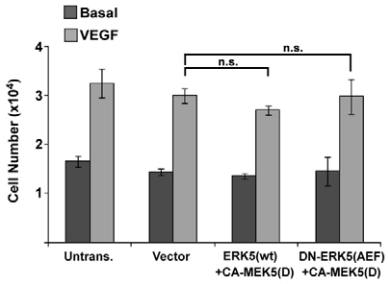

Fig. 6. Co-transfection of ERK5 and constitutively active MEK5(D) does not stimulate HDMEC proliferation. HDMECs were transiently transfected with 2.5 μg ERK5(wt) and 2.5 μg CA-MEK5(D), 2.5 μg DN-ERK5(AEF) + 2.5 μg CA-MEK5(D) or mock-transfected with 5.0 μg empty pcDNA3.1 (Vector). 24 hours after transfection, HDMECs were seeded at 1.5×10^4 cells/well on gelatin-coated 24-well plates. 24 hours after seeding, cells were serum starved for 20 hours, before stimulation with VEGF (50 ng/ml). 72 hours after stimulation, cell numbers were quantified by CellTiter-Glo® luminescence assay. Data are presented as cell number (n=3; mean ± s.d.; n.s., not significant).

absence of VEGF (Fig. 5). Co-expression of DN-ERK5(AEF) and CA-MEK5(D) did not stimulate ERK5 phosphorylation, AKT phosphorylation, BAD phosphorylation or BCL2 expression under basal conditions. Furthermore, DN-ERK5(AEF) and CA-MEK5(D) co-expression blocked VEGF-stimulated ERK5 phosphorylation, AKT phosphorylation and BAD phosphorylation. VEGF-mediated increase in BCL2 expression and suppression of cleaved caspase-3 was also blocked by co-expression of DN-ERK5(AEF) and CA-MEK5(D) (Fig. 5). Taken together, these results confirm our previous biochemical analysis with siRNA-mediated silencing of ERK5, in that ERK5 regulates activation of AKT and the downstream activation of BAD, resulting in suppression of caspase-3 activity, which ultimately leads to HDMEC survival on a collagen matrix.

Constitutive activation of ERK5 stimulates and sustains VEGF-induced tubular morphogenesis

In accordance with the lack of effect of siRNA-mediated downregulation of *ERK5* upon HDMEC proliferation, co-transfection of HDMECs with either ERK5(wt) and CA-MEK5(D), or co-transfection of DN-ERK5(AEF) and CA-MEK5(D) had no effect upon VEGF-stimulated cellular proliferation (Fig. 6). Co-transfection of ERK5(wt) and CA-MEK5(D) stimulated HDMEC tubular morphogenesis in the absence of VEGF over 24 hours (Fig. 7A,B) and sustained VEGF-stimulated tubular morphogenesis for 5 days (Fig. 7C,D). Normal VEGF-stimulated tubular morphogenesis in this assay is maximal after 24 hours, and tubes rapidly regress after this time period; therefore the effect of sustaining VEGF-induced tube formation for 5 days was significant. By contrast, co-transfection of HDMECs with DN-ERK5(AEF) and CA-MEK5(D), did not stimulate tubular morphogenesis under basal conditions, and blocked VEGF-stimulated morphogenesis over 24 hours and 5 days (Fig. 7A–C). Together, these data demonstrate that ERK5 activity is essential for VEGF-stimulated tube formation, but is dispensable for VEGF-stimulated proliferation of HDMECs.

ERK5 activity is required for VEGF-stimulated angiogenesis in a HDMEC and NHDF co-culture assay

The ability of ERK5 to regulate tubular morphogenesis in HDMECs in a collagen gel suggested that ERK5 has the potential to regulate VEGF-stimulated angiogenesis. We used an endothelial and fibroblast co-culture assay to model events in a more representative assay of in vivo angiogenesis (Donovan et al., 2001; Sorrell et al., 2007). HDMECs were plated on a monolayer of normal human dermal fibroblasts (NHDFs) and either left unstimulated or stimulated with VEGF over a period of 5 days. The cells were then stained for expression of the endothelial-specific marker CD31 and visualised by immunohistochemistry. Quantification of the tubular networks showed that VEGF-stimulation of co-cultures resulted in a threefold increase in the endothelial tubular network after 5 days (Fig. 8A,B). siRNA-mediated silencing of either *ERK5* or *MEK5*, using two individual duplexes directed against each target, resulted in a profound inhibition of both basal, and VEGF-stimulated tubule formation (Fig. 8A,B). The expression, and efficient siRNA-mediated knockdown of ERK5, in both NHDFs and HDMECs after 5 days was confirmed by western blotting (Fig. 8C). Analysis of cleaved caspase-3 levels in HDMEC and NHDF co-cultures by immunofluorescence revealed that siRNA-mediated silencing of *ERK5* resulted in an increase in cleaved caspase-3, which co-localised with the CD31-positive HDMECs within the co-culture (Fig. 8D). Taken together, these results not only confirm our previous observation in collagen gels that ERK5 is required for VEGF-stimulated tubular morphogenesis, but also suggest that

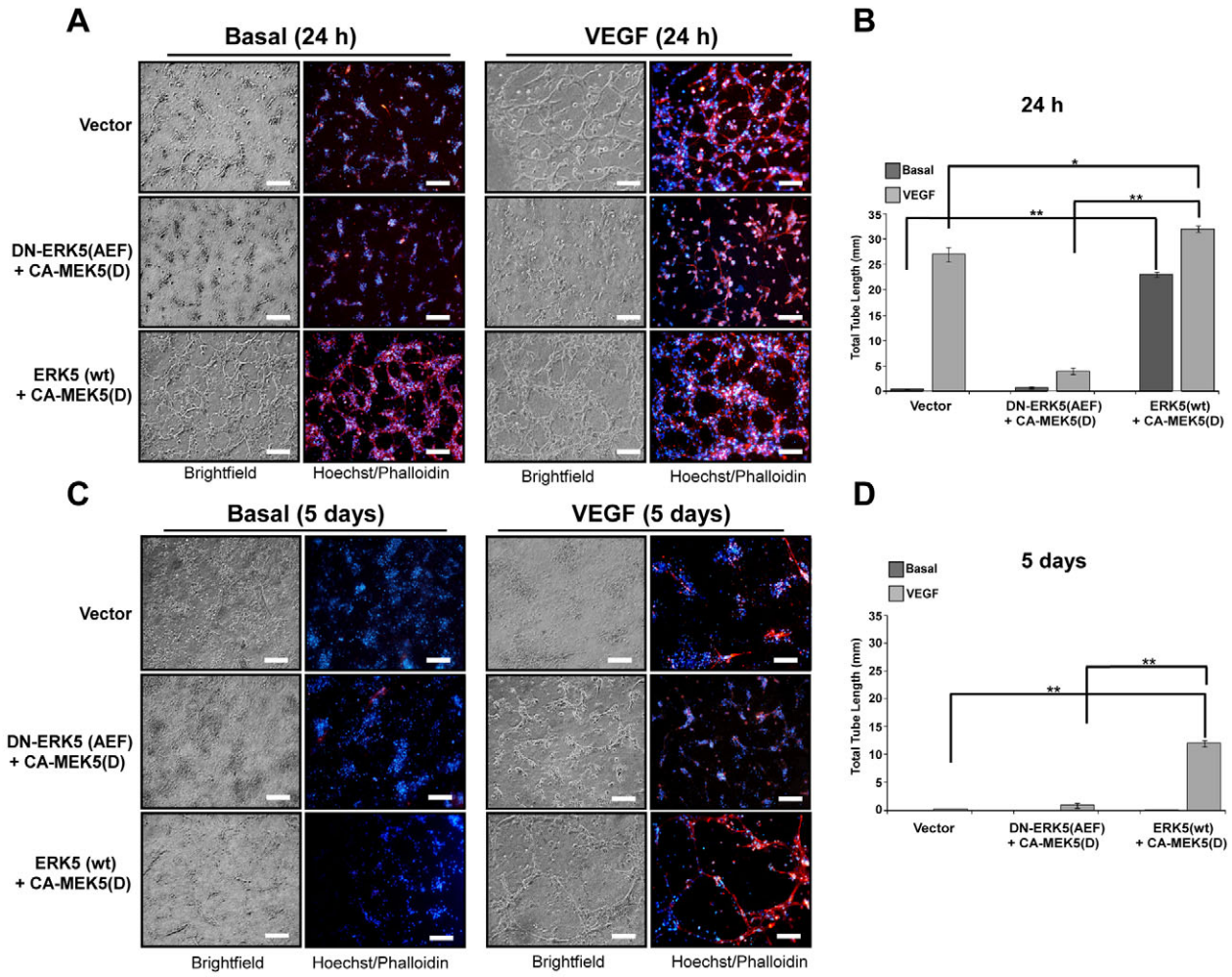

Fig. 7. Co-transfection of ERK5 and constitutively active MEK5(D) stimulates and sustains tubular morphogenesis in HDMEC. HDMECs were transiently transfected with 2.5 µg ERK5(wt) + 2.5 µg CA-MEK5(D), 2.5 µg DN-ERK5(AEF) + 2.5 µg CA-MEK5(D) or mock-transfected with 5.0 µg empty pcDNA3.1 (Vector). 24 hours after transfection, HDMECs were serum starved for 20 hours, and seeded at 1.25×10^5 cells per well between two layers of collagen I, before stimulation with VEGF (50 ng/ml). 24 hours (**A**) and 5 days (**C**) after VEGF stimulation, cells were fixed, and the actin stress fibres and nuclei were stained with Rhodamine-coupled Phalloidin (red) and Hoechst 33342 (blue), respectively. Results are from one experiment, which is representative of three. (**B**,**D**) The total length of tubular structures was quantified from three fields (×10 objective) per well (n=3; mean ± s.d.; *P<0.05, **P<0.01). Scale bars: 100 µm.

endothelial cells have a specific requirement for ERK5 to suppress caspase-3 activation and ultimately facilitate VEGF-mediated survival and tubular morphogenesis in endothelial cells.

Discussion

Angiogenesis is a complex, multi-faceted process that requires the coordinated migration, proliferation and ultimate differentiation of endothelial cells to form a lumen-containing tubular vessel capable of allowing blood flow. We have used a model system to analyse the role of VEGF-mediated ERK5 activity in HDMECs during proliferation and differentiation. Our results are the first to reveal that ERK5 is specifically required for VEGF-mediated tubular morphogenesis, but is dispensable for cellular proliferation in primary HDMECs. We further define a specific role for ERK5 in the regulation of VEGF-mediated AKT phosphorylation and the suppression of caspase-3 activity during tubular morphogenesis, revealing that ERK5 regulates a defined component of VEGF-stimulated angiogenesis in endothelial cells.

VEGF stimulates a number of intracellular signalling pathways that regulate cellular proliferation, migration and survival (Holmes et al., 2007). VEGF has been reported to stimulate ERK5 activity in human umbilical vein endothelial cells (HUVECs) and mouse lung capillary endothelial cells (MLCECs) (Hayashi et al., 2004). However, the potential role of ERK5 in the mediation of VEGF-induced physiological responses had not been previously explored. In contrast to ERK1/2, VEGF-mediated activation of ERK5 was sustained under conditions of both proliferation and differentiation, with a very similar kinetic profile of activation (Fig. 1C,D). Reduction of ERK5 levels by siRNA-mediated gene silencing, revealed a specific role for ERK5 in the regulation of VEGF-mediated HDMEC tubular morphogenesis (Fig. 2C), but not proliferation (Fig. 2B). siRNA-mediated silencing of *MEK5* also prevented tubular morphogenesis (supplementary material Fig. S2). The importance of ERK5 in the specific regulation of tubular morphogenesis was highlighted by the finding that co-expression of constitutively active MEK5, together with ERK5, stimulated tubular morphogenesis in

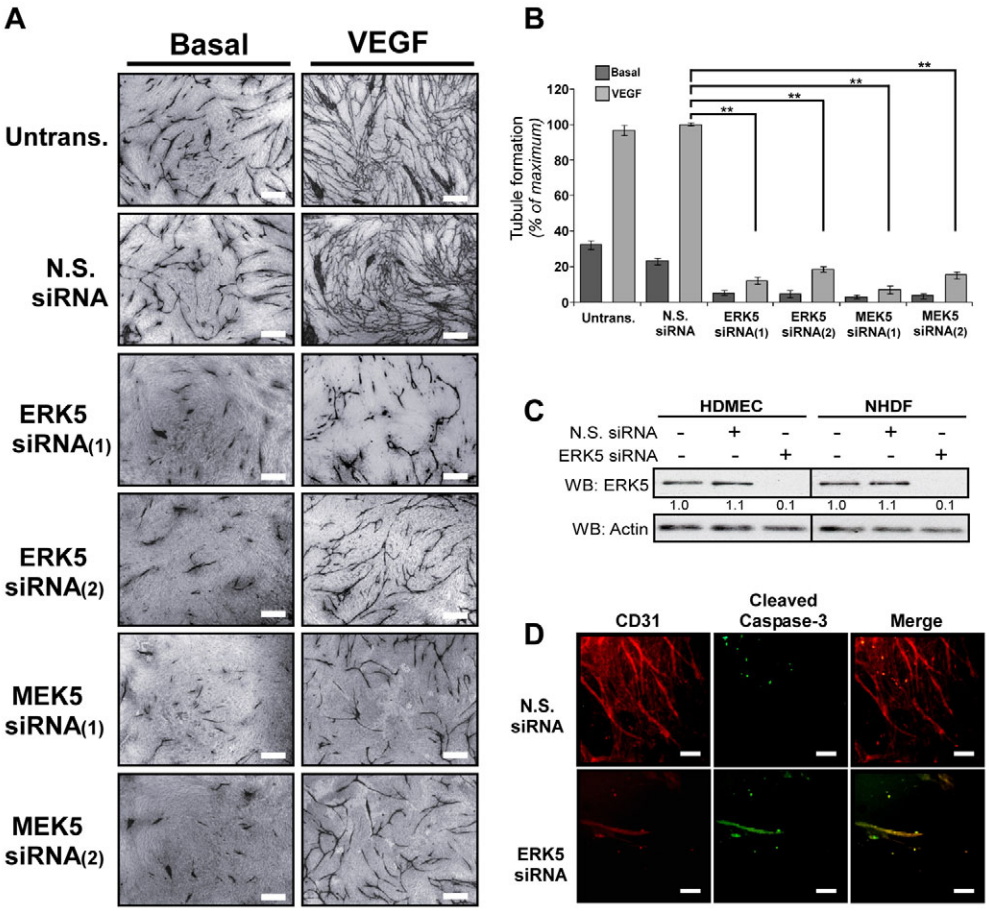

Fig. 8. Downregulation of ERK5 expression prevents VEGF-stimulated angiogenesis in a HDMEC and NHDF co-culture assay. HDMECs were transfected with 10 nM *ERK5* siRNA1, 10 nM *ERK5* siRNA2, 10 nM non-silencing (N.S.) siRNA or left untransfected (Untrans.). (**A**) 24 hours after transfection, HDMECs were plated onto a confluent NHDF monolayer, and treated with VEGF (50 ng/ml) or left unstimulated (Basal) with or without siRNA. (**B**) Total tubule area was quantified by AngioQuant software. Data are presented as a percentage of maximum tube formation (n=3; mean ± s.d.; $^{**}P<0.01$). (**C**) 48 hours after transfection of NHDFs or HDMECs with *ERK5* siRNA (5 nM *ERK5* siRNA1 + 5 nM *ERK5* siRNA2), or with non-silencing (N.S.) siRNA (10 nM), cells were lysed and separated on a 10% SDS-PAGE gel followed by western blotting (WB) with antibodies directed against ERK5 and actin, as indicated. Expression of ERK5 relative to actin was quantified by densitometric analysis. (**D**) HDMEC and NHDF co-cultures transfected with *ERK5* siRNA (10 nM) or with N.S. siRNA (10 nM), and stimulated with VEGF were fixed and stained with anti-CD31 (red) or with cleaved caspase-3 (Asp175) antibody Alexa Fluor® 488 conjugate (green). Scale bars: 400 μm. Results are from one experiment, which is representative of three.

the absence of VEGF, and significantly sustained VEGF-stimulated tubular morphogenesis (Fig. 7A–D). Activation of ERK5 has been previously shown to regulate the differentiation of myoblasts (Dinev et al., 2001), by regulating the pro-myogenic actions of IGF-2 (insulin-like growth factor 2) (Carter et al., 2009), suggesting that ERK5 regulates the differentiation of a number of cell types.

The lack of any effect upon manipulation of ERK5 activity in HDMECs, either by siRNA-mediated gene silencing (Fig. 2B) or overexpression (Fig. 6), after VEGF-mediated proliferation, suggests that in these cells, ERK5 activity is dispensable for this response. By contrast, knockdown of ERK1/2 clearly affected VEGF-mediated proliferation (supplementary material Fig. S1D), in agreement with the established role of ERK1/2 in the regulation of cell cycle entry (Brunet et al., 1999). The role of ERK5 in regulating cell proliferation remains unclear. Although epidermal growth factor (EGF)-mediated proliferation of fibroblasts (Kato et al., 1998) and GM-CSF (granulocyte-macrophage colony-stimulating factor)-mediated proliferation of haematopoietic cells (Dong et al., 2001) are reported to be ERK5 dependent, other research using cells from *Erk5*$^{-/-}$ and *Mek5*$^{-/-}$ mice has revealed that ERK5 and MEK5 are not required for cell cycle progression (Hayashi et al., 2004; Wang et al., 2005). The sensitivity of cells on a collagen matrix to ERK5 downregulation is reflected in the increased loss of VEGF-mediated activation of both AKT and BAD on this matrix, following ERK5 silencing, compared with a gelatin matrix (Fig. 3A). Activation of AKT requires its binding to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] via the pleckstrin homology domain and phosphorylation at Thr308 in the activation loop and phosphorylation at Ser473 in the C-terminus (Alessi et al., 1996; Stokoe et al., 1997). The mammalian target of rapamycin complex 2 (mTORC2) phosphorylates AKT at Ser473 (Jacinto et al., 2006; Sarbassov et al., 2005), whereas 3′-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates AKT at Thr308 (Alessi et al., 1996). VEGF-stimulated AKT activation is regulated by phosphatidylinositol 3-kinase (PI3K) (Gerber et al., 1998b; Thakker et al., 1999). However, VEGF-mediated ERK5

activation was not inhibited by either the PI3K inhibitor wortmannin, or the mTOR inhibitor rapamycin (supplementary material Fig. S3), suggesting that ERK5 is not downstream of either PDK1 or mTORC2. Furthermore, the ability of both wortmannin and chronic exposure to rapamycin (Sarbassov et al., 2006) to inhibit VEGF-mediated phosphorylation of AKT and BAD, without inhibiting ERK5 activity, confirmed that the classical PI3K–PDK1 and mTORC2 pathways are able to regulate AKT phosphorylation and that AKT phosphorylation is required for the subsequent phosphorylation of BAD (supplementary material Fig. S3). The precise mechanism of ERK5-mediated regulation of AKT activity remains obscure. It is possible that ERK5 regulates the activity of a phosphatase, such as PP2A, which has been shown to interact with β1 integrin and dephosphorylate AKT at both Ser473 and Thr308 (Pankov et al., 2003). The recent finding that ERK5 immunoprecipitates with integrins β1, αvβ3 and αvβ5 in breast cancer cells (Sawhney et al., 2009), raises the possibility that ERK5 binds to specific integrins to coordinate signals from both the cell matrix and VEGFR-2 to regulate AKT phosphorylation and facilitate tubular morphogenesis in endothelial cells.

A role for Erk5 in the regulation of Akt phosphorylation at Ser473 and Thr308 has previously been reported in mouse embryonic fibroblasts under conditions of osmotic stress (Wang et al., 2006). In mouse neuronal cells, nerve growth factor (NGF)-mediated phosphorylation of Akt on Ser473, but not Thr308, is dependent upon ERK5 activity (Finegan et al., 2009). Recently, it has been shown that Erk5 regulates PDGF-stimulated Akt phosphorylation at Ser473 in porcine aortic endothelial (PAE) cells (Lennartsson et al., 2010). This suggests that ERK5 regulates AKT activity in response to various agonists in different cells.

Activation of AKT is important for the regulation of apoptosis because it has been shown to phosphorylate the pro-apoptotic protein BAD on Ser136, leading to its cytosolic sequestration by 14-3-3 protein (Datta et al., 1997). Furthermore, AKT also regulates expression of the anti-apoptotic protein BCL2 (Pugazhenthi et al., 2000). BCL2 and BAD heterodimerise and neutralise each others' function. BCL2 regulates apoptosis by preventing the release of cytochrome c from the mitochondria and the sequential activation of the initiator caspase-9 and terminal executioner caspase-3 pathway, ultimately resulting in endonuclease-mediated degradation of DNA, cytoplasmic and chromatin condensation and cell death (Cory et al., 2003). Consistent with the role of AKT in regulating apoptosis, we found that knockdown of ERK5 prevented VEGF-mediated phosphorylation of BAD (Fig. 3A) and also prevented expression of *BCL2* mRNA in HDMECs plated in a collagen gel (Fig. 3B). ERK5 knockdown also induced caspase-3 activation on this matrix (Fig. 4A–H). Conversely, constitutive activation of ERK5 resulted in increased phosphorylation of BAD, increased expression of BCL2 and suppressed activation of caspase-3 (Fig. 5). VEGF has previously been reported to increase BCL2 levels in HUVECs (Gerber et al., 1998a) and to enhance HDMEC survival in collagen gels (Nor et al., 1999), whereas increased BAD phosphorylation has been shown to prevent apoptosis in rat sinusoidal endothelial cells (Ohi et al., 2006). Our data show that ERK5 is crucial for regulation of the apoptotic balance of these signalling molecules.

Gene ablation of *Erk5* in mice results in embryonic lethality at around E10.5 as a result of abnormal cardiovascular development (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003). Histological analysis of the *Erk5*-null embryos has revealed that the vascular endothelial cells are irregularly aligned, and undergo apoptosis, resulting in defective angiogenesis. Previous data has also shown that ERK5 is required to protect endothelial cells against fluid shear stress (Pi et al., 2004). The requirement for ERK5 activity to protect endothelial cells from apoptosis has been ascribed to the need for ERK5-mediated phosphorylation of the myocyte enhancer factor 2C (MEF2C) transcription factor (Hayashi et al., 2004). This is based on the fact that the phenotype of *Mef2c*$^{-/-}$ mice is similar to that of *Erk5*$^{-/-}$mice, with embryonic lethality resulting from cardiac and vascular malformations (Hayashi and Lee, 2004; Roberts et al., 2009). However, infection of *Erk5*$^{-/-}$ embryos with an adenovirus encoding a constitutively active Mef2c was only able to partially protect endothelial cells from apoptosis (Hayashi et al., 2004), suggesting the existence of additional effectors downstream of ERK5 that regulate apoptosis (Olson, 2004).

Our data reveal that ERK5 is able to regulate VEGF signalling in human endothelial cells, providing a mechanism for the adverse effects on angiogenesis seen in murine *Erk5*$^{-/-}$ embryos. Conditional knockouts of *Erk5* in specific cell types including cardiomyocytes, hepatocytes and neuronal cells develop normally (Hayashi and Lee, 2004), suggesting that endothelial cells have a particular requirement for ERK5. Current anti-angiogenic drug therapy is centred around the development of inhibitors that target the VEGF ligand or VEGFR-2 (Holmes et al., 2007), which inhibit all the actions of VEGF on endothelial cells. Our results, showing that ERK5 specifically regulates tubular morphogenesis rather than proliferation, suggest that drugs targeting the ERK5 pathway in vivo may uniquely allow selective interference of a specific component of angiogenesis.

Materials and Methods

Cell culture

HDMECs and NHDFs were purchased from Promocell (Heidelberg, Germany). All cells were routinely grown on gelatin-coated cell culture dishes [1% w/v gelatin from porcine skin (Sigma, Poole, UK)]. HDMECs at passages (p) 2–8 were grown in EBM MV2 growth medium (Promocell) containing 5% v/v foetal calf serum (FCS), EGF (5 ng/ml), hydrocortisone (0.2 μg/ml), VEGF (0.5 ng/ml), FGF-2 (10 ng/ml), insulin-like growth factor-1 (20 ng/ml) and ascorbic acid (1 μg/ml). NHDFs (p 4–12) were grown in fibroblast growth medium (Promocell) supplemented with FGF-2 (1 ng/ml) and insulin (5 μg/ml). All cells were incubated at 37°C in humidified air containing 5% v/v CO_2.

Cell stimulation and preparation of cell lysates

Before stimulation with VEGF, HDMECs were serum-starved for 20 hours in EBM MV2 basal medium containing 1% (v/v) FCS. Cells were stimulated with 50 ng/ml VEGF-A_{165} (Peprotech, Rocky Hill, NJ), washed in ice-cold Dulbecco's phosphate-buffered saline (DPBS) (Lonza, Basel, Switzerland) and lysed on ice in modified RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonylfluoride). Lysates were clarified by centrifugation (23,000 ***g*** for 20 minutes at 4°C). For detection of cleaved caspase-3, cells were lysed in LDS sample buffer (Invitrogen, Paisley, UK) containing 5% (v/v) β-mercaptoethanol (Sigma). Protein extracts were sonicated for 10 seconds using a Soniprep 150 sonicator (Sanyo-Gallenkamp, Leicester, UK) before heat denaturation at 90°C for 5 minutes.

Western blot analysis

Proteins were resolved by SDS-PAGE on NuPAGE® 10% or 4–12% pre-cast gradient gels (Invitrogen) and transferred onto Hybond-C Extra nitrocellulose membranes (GE Healthcare, Amersham, UK). Membranes were blocked using 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline containing 0.01% (v/v) Tween 20 (TBST). Membranes were probed overnight with primary antibodies, diluted in TBST containing 2% (w/v) BSA, or 5% (w/v) non-fat milk, directed against: ERK5; AKT; Flag-Tag (DYKDDDDK Tag), HA-Tag (6E2), phospho-VEGFR-2 (Tyr1175); phospho-ERK5 (Thr218/Tyr220); phospho-p44/42 MAP kinase (Thr202/Tyr204); phospho-AKT (Thr308); phospho-AKT (Ser473); phospho-p70 S6 kinase (Thr389)(108D2); phospho-BAD (Ser136); Bcl-2 (Human Specific); and cleaved caspase-3 (Asp175) (New England Biolabs, Hitchin, UK). A primary antibody directed against MEK5 (H-94) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody directed against Actin (C-11) or Lamin B1 (H-90) (Santa

Cruz Biotechnology) was used to assess protein loading in all western blot experiments. Proteins were detected using rabbit-specific secondary antibodies and ECL (enhanced chemiluminescence) western blotting detection reagents (GE Healthcare).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted using the RNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Harvested RNA was treated with DNaseI (Qiagen) before first-strand cDNA synthesis carried out by reverse transcription of 1 μg total RNA using oligo(dT)$_{18}$ and M-MLV Reverse Transcriptase (Invitrogen). Reaction mixtures for qRT-PCR contained 1.5 μl cDNA template, 4.5 μl distilled H_2O, 10 μl 2× Power SYBR® Green mastermix (Applied Biosystems, Warrington, UK) and 0.25 μM forward and reverse primers (Invitrogen) in each case. Primers used were: *ERK5*, Hs_MAPK7_1_SG QuantiTect Primer Assay (Qiagen); *BCL2* forward, 5′-GTC TGG GAA TCG ATC TGG AA-3′, and *BCL2* reverse, 5′-GCA ACG ATC CCA TCA ATC TT-3′; β-*actin* forward, 5′-ATG GAT GAT GAT ATC GCC GC-3′ and β-*actin* reverse, 5′-AAG CCG GCC TTG CAC AT-3′. qRT-PCR reactions were performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following parameters: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Cycle threshold (C_T) values for the mRNA of interest and for the β-*actin* control were recorded, and changes in gene expression were determined by the Comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

Transfection of siRNA

ERK5-specific short interfering RNA (siRNA) duplexes directed against the target sequences 5′-CAG ACC CAC CTT TCA GCC TTA-3′ for Hs_MAPK7_9_HP (designated *ERK5* siRNA1) and 5′-CAG GAT GGC CAG GCA GAT TCA-3′ for Hs_MAPK7_5_HP (designated *ERK5* siRNA2), and *MEK5*-specific siRNA directed against the target sequences 5′-AAG ACG TAT GTT GGA ACA AAT-3′ for Hs_MAP2K5_11_HP (designated *MEK5* siRNA1) and 5′-CAA GAC GTA TGT TGG AAC AAA-3′ for Hs_MAP2K5_12_HP (designated *MEK5* siRNA2), ERK1- and ERK2-specific siRNA duplexes, directed against the target sequences 5′-CTC CCT GAC CCG TCT AAT ATA-3′ for Hs_MAPK3_7 HP (designated *ERK1* siRNA) and 5′-AAG TTC GAG TAG CTA TCA AGA-3′ for HS_MAPK1_10 HP (designated *ERK2* siRNA) and a non-silencing (N.S.) control siRNA, directed against the sequence 5′-AAT TCT CCG AAC GTG TCA CGT-3′, were purchased from Qiagen. All siRNA duplexes were validated by qRT-PCR and western blotting prior to use (Holmes et al., 2010). HDMECs were seeded at 1×10^5 cells per well, on gelatin-coated six-well dishes in 2 ml EBM MV2 growth medium, and incubated for 24 hours. Transfection was carried out in a total volume of 2.5 ml per well of OptiMEM medium (Gibco, Paisley, UK) containing 10 nM siRNA and 0.1% (v/v) Lipofectamine™ RNAiMAX (Invitrogen). Cells were incubated with the transfection mix for 4 hours before washing in DPBS containing Ca^{2+} and Mg^{2+} and the addition of EBM MV2 growth medium. Cells were incubated for a further 24 hours before lysis and immunoblotting. Alternatively, after washing, HDMECs were serum-starved overnight in EBM MV2 basal medium containing 1% (v/v) FCS for use in cell-based assays..

Plasmid constructs

Wild-type Flag-tagged ERK5 (pcDNA3.1BMK1); dominant-negative Flag-tagged ERK5 [pcDNA3.1BMK1(AEF)] and constitutively active HA-tagged, MEK5 [pCMV5MEK5(D)] plasmids were kindly donated by Prof. Jiing-Dwan Lee (Scripps Research Institute, La Jolla, CA) (Kato et al., 1997). Constitutively active HA-tagged MEK5 was digested from pCMV5MEK5(D) with *Xba*I and *Kpn*I (New England Biolabs) and subcloned into pcDNA3.1 (Invitrogen).

Transfection of plasmid constructs

HDMECs were seeded on six-well gelatin-coated cell culture plates at 9.3×10^4 and incubated for 24 hours. Growth medium was replaced with 2 ml fresh EBM MV2 growth medium immediately before transfection. Plasmid DNA (2.5 μg) was mixed with serum-free OptiMEM medium and transfected using TransPass™ HUVEC Transfection Reagent (New England Biolabs) according to the manufacturer's instructions. Cells were incubated with transfection mix for 4 hours, washed twice in DPBS followed by the addition of EBM MV2 growth medium. Cells were incubated for 24 hours, and serum starved in EBM MV2 medium containing 1% (v/v) FCS before use in assays.

3D collagen gel tube formation assay

PureCol™ type I collagen was purchased from Inamed Biomaterials (Leimuiden, The Netherlands). Collagen gels were prepared as described previously (Bohman et al., 2005). Serum-starved HDMECs were seeded at 1.25×10^5 cells per well of a 24-well plate before the addition of the top layer of collagen, and stimulation with VEGF (50 ng/ml) for 20 hours. Length of tubular structures was quantified using NIH ImageJ software as described previously (Matsumoto et al., 2002).

HDMEC and NHDF co-culture angiogenesis assay

NHDFs were seeded in fibroblast growth medium at 1.5×10^4 cells per well on gelatin-coated 24-well plates and incubated for 3 days. On day 4, HDMECs treated with siRNA, or not, were seeded at 4.5×10^3 cells per well, on top of the NHDF monolayer in EBM MV2 growth medium. On day 5, co-cultures were transfected with siRNA (as described above) for 4 hours, followed by washing in DPBS, and replacement of EBM MV2 growth medium. On day 6, cells were placed in EBM MV2 basal medium containing 1% (v/v) FCS with, or without, 50 ng/ml VEGF. On days 7 and 9, co-cultures were treated with siRNA, as described above. On day 8, cells were placed in fresh EBM MV2 medium containing 1% (v/v) FCS with, or without, 50 ng/ml VEGF. On day 10, co-cultures were fixed in ice-cold 70% (v/v) ethanol for 30 minutes before addition of blocking buffer (1% (w/v) BSA in DPBS) for 30 minutes. Monoclonal mouse anti-human CD31 antibody, (Dako Cytomation, Glostrup, Denmark) (1:750 dilution in blocking buffer) was added for 1 hour at 37°C, followed by three washes in blocking buffer. Anti-mouse IgG (whole molecule) alkaline-phosphatase-conjugated antibody (Sigma) was diluted 1:200 in blocking buffer and incubated for 1 hour at 37°C. Tubes formed from endothelial cells were visualised by the addition of the substrate SigmaFast™ BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) solution (Sigma).

Quantification of tube formation in HDMEC and NHDF co-cultures

Tube formation in co-culture plates was analysed by photographing three randomly selected fields of view, in triplicate wells per condition, at low magnification (×4 objective) using a Nikon Eclipse TS 100 microscope attached to a Nikon DS-Fi1-L2 digital camera (Nikon, Kingston upon Thames, UK). Total tubule length was quantified using AngioQuant software (http://www.cs.tut.fi/sgn/csb/angioquant/) (Niemisto et al., 2005).

Immunostaining

HDMECs undergoing tubular morphogenesis in 3D collagen gels were fixed with paraformaldehyde and stained with Phalloidin as described previously (Mellberg et al., 2009). When detecting cleaved caspase-3 within co-cultures, fixed co-cultures were blocked in blocking buffer [5% (v/v) normal goat serum (Vector Laboratories, Burlingame, CA) in DPBS containing 0.3% (v/v) Triton X-100] at 37°C for 30 minutes. Monoclonal mouse anti-human CD31 antibody (Dako Cytomation) was diluted 1:750, together with a 1:10 dilution of cleaved caspase-3 (Asp^{175}) antibody Alexa Fluor® 488 conjugate (New England Biolabs) in 1% (w/v) BSA in DPBS containing 0.2% (v/v) Triton X-100. Cells were incubated with the antibody solution overnight, in the dark, at 4°C with gentle rocking. Cells were washed, and incubated with Alexa Fluor® 568 goat anti-mouse IgG (Molecular Probes) diluted 1:200 in 1% (w/v) BSA in DPBS, in the dark, at room temperature for 2 hours. Finally, cells were washed in ddH_2O, and analysed using a Nikon TE2000 inverted fluorescence microscope.

Cell proliferation assay

HDMECs were seeded at 1.5×10^4 cells per well in 24-well plates in EBM MV2 growth medium. After 24 hours, cells were washed in DPBS and serum-starved in 1 ml EBM MV2 medium containing 1% (v/v) FCS for 20 hours. Cells were stimulated, or not, with VEGF (50 ng/ml) and incubated for a further 72 hours. Cell numbers were quantified using a Cell Titer-Glo™ luminescent cell viability assay reagent (Promega, Southampton, UK), as previously described (Crouch et al., 1993). Luminescence was measured on a Wallac Victor^2 1420 Multilabel Counter (Perkin Elmer Life Sciences, Fremont, CA).

Cell cycle analysis

Determination of the cell cycle distribution was carried out by FACS analysis as described previously (Mellberg et al., 2009).

Annexin V apoptosis assay

Early apoptosis of HDMECs was detected as described previously (Dimberg et al., 2008).

Fluorogenic caspase-3/7 assay

HDMECs on gelatin or collagen matrices in 24-well plates were washed briefly in DPBS before complete cell lysis in Caspase-Glo® 3/7 reagent (Promega). Aliquots of the resultant lysates were transferred into separate wells of black-walled 96-well plates, and luminescence was measured using a Wallac Victor^2 1420 Multilabel Counter.

Signal transduction inhibitors

Rapamycin and wortmannin were purchased from Calbiochem (Merck Chemicals Ltd, Nottingham, UK) and reconstituted in DMSO (Sigma). Cells were pre-incubated with indicated amounts of signal transduction inhibitors for the times shown in EBM MV2 basal medium containing 1% (v/v) FCS at 37°C, before stimulation with VEGF.

Statistical analysis

Results are expressed as mean ± s.d. Statistical analyses using an unpaired Student's *t*-test were performed using SPSS (version 16.0), with $P<0.05$ considered to be significant.

This study was supported by a CASE studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) and AstraZeneca (O.Ll.R.); the North West Cancer Research Fund (NWCRF) (K.H., J.M. and M.J.C.).

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/18/3189/DC1

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