The role of ERK5 in endothelial cell function

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Abbreviations: BLMEC, bovine lung microvascular endothelial cell; DUSP, dual-specificity phosphatase; E, embryonic day; eNOS, endothelial nitric oxide synthase; ERK, extracellularsignal-regulated kinase; FGF2, fibroblast growth factor 2; HDMEC, human dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; KLF, Krüppel-like factor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEF, myocyte enhancer factor; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MKP, MAPK phosphatase; NF-κB, nuclear factor κB; NLS, nuclear localization signal; NRF2, nuclear erythroid 2-related factor 2; PKB, protein kinase B; RSK, ribosomal S6 kinase; SGK, serum- and glucocorticoid-regulated protein kinase; TNFα, tumour necrosis factor α; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

Abstract
Extracellular-signal-regulated kinase 5 (ERK5), also termed big MAPK1 (BMK1), is the most recently discovered member of the mitogen-activated protein kinase (MAPK) family. It is expressed in a variety of tissues and is activated by a range of growth factors, cytokines and cellular stresses. Targeted deletion of *Erk5* in mice has revealed that the ERK5 signalling cascade is critical for normal cardiovascular development and vascular integrity. *In vitro* studies have revealed that, in endothelial cells, ERK5 is required for preventing apoptosis, mediating shear-stress signalling and regulating tumour angiogenesis. The present review focuses on our current understanding of the role of ERK5 in regulating endothelial cell function.

Introduction
Mitogen-activated protein kinases (MAPKs) play an essential role in regulating many cellular processes including growth, differentiation and apoptosis. MAPKs are activated by a range of growth factors and chemical stimuli such as oxidative stress and osmotic imbalance, and are responsible for transducing extracellular signals to the cytoplasm and nucleus. In mammalian cells, the MAPK signalling system consists of four distinct linear signalling cascades terminating in: extracellular-signal-regulated kinase (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) 1, 2 and 3, p38 MAPKs (p38 *α*,*β*,*γ* and *δ*), or the most recently discovered MAPK, ERK5, also termed big MAPK1 (BMK1) [1,2]. Each of these terminal kinases phosphorylates a range of cellular targets from cytoplasmic enzymes to transcription factors [3].

Structure of ERK5
The human *ERK5* gene (also termed *MAPK7*) is present on chromosome 17p11.2 and spans 5824 bases. It has an ORF of 2451 bp encoding a protein of 816 amino acids with a predicted molecular mass of 102 kDa (Figure 1). ERK5 shares 66% sequence homology with ERK2 within the kinase domain, which contains the Thr-Glu-Tyr (TEY) dual phosphorylation motif in the activation loop [4]. The N-terminal domain (amino acids 1–406) of ERK5 contains a region important for cytoplasmic targeting (amino acids 1–77) and the kinase domain (amino acids 78–406), which can be further separated into regions required for interaction with MAPK/ERK kinase 5 (MEK5) (amino acids 78–139) and oligomerization (amino acids 140–406) [5]. The large size of ERK5 is attributable to its long C-terminal tail of 410 amino acids, which is unique among the MAPKs. The C-terminal domain contains a nuclear localization signal (NLS) (amino acids 505–539) situated between two proline-rich domains (amino acids 434–465, amino acids 578–701) that are proposed to serve as binding sites for Src homology 3 (SH3)-domain containing proteins [4,5].The C-terminal region also contains a myocyte enhancer factor 2 (MEF2)-interacting region (amino acids 440–501) and a transcriptional activation domain (amino acids 664–789), which regulates MEF2 transcription factor activity [6]. Truncation of the C-terminal tail results in increased ERK5 kinase activity, revealing that the C-terminal tail of ERK5 also has an autoinhibitory function [7].

Activation and regulation of the ERK5 signalling axis
Activation of a MAPK signalling module is initiated by the activation of the apical MAPK kinase kinase (MAPKKK) by an extracellular stimulus, resulting in the sequential activation of the MAPK kinase (MAPKK) and ultimately the MAPK [8]. MEK5 is the upstream MAPKK that specifically phosphorylates ERK5 on Thr218/Tyr220 within the TEY motif in the activation loop, resulting in an increase in the catalytic activity of ERK5 [4,9,10] (Figure 1). MEK5 is activated by the upstream MAPKKKs, MEK kinase 2 (MEKK2) and MEKK3, which specifically phosphorylate Ser313/Thr317 in MEK5 [11,12].

Active ERK5 is able to phosphorylate MEK5 [13] as well as autophosphorylate on a number of C-terminal tail residues, leading to an enhancement of ERK5 transcriptional activity [14]. More recent data have shown that ERK5 is able to undergo MEK5-independent phosphorylation by cyclin-dependent kinases (CDKs), revealing a potential novel mode of ERK5 activation [15,16]. Dephosphorylation of MAPKs on the Thr-Xaa-Tyr (TXY) motif by a MAPK phosphatase (MKP) subfamily of dual specificity phosphatases (DUSPs) leads to their inactivation [17]. Currently,

no DUSP has been identified that dephosphorylates ERK5. However, ERK5 is dephosphorylated by the phosphotyrosine-specific phosphatase PTP-SL, which interacts with ERK5 and impedes its translocation to the nucleus [18]. ERK5 is also regulated by post-translational modifications; it has recently been reported that ERK5 undergoes SUMOylation by small ubiquitin-related modifier 3 (SUMO3) on Lys6 and Lys22 following treatment with H2O2 and advanced glycation end-products (AGE) in human umbilical vein endothelial cells (HUVECs) [19].

Similar to the other MAPKs, ERK5 belongs to a family of evolutionarily conserved proline-directed protein kinases that phosphorylate substrates on serine and threonine residues immediately preceding a proline residue. However, certain serine and threonine autophosphorylation sites in ERK5 are not followed by proline [13,14], suggesting that the specificity of ERK5 may differ from other MAPK family members or that another kinase may be involved in the phosphorylation of these sites. Activation of the ERK5 signalling axis stimulates both distinct and similar pathways to the classical ERK1/2 pathway [20]. Downstream targets of ERK5 include the MEF family of transcription factors: MEF2A, MEF2C and MEF2D, Sap-1a (serum-response factor accessory protein 1a; ETS domain transcription factor), c-Myc, serum- and glucocorticoid-regulated protein kinase (SGK) and p90 ribosomal S6 kinase (RSK) (reviewed in [21,22]).

The role of ERK5 *in vivo*

To address the physiological role of the ERK5 signalling axis, researchers have utilized gene targeting in mice to ablate specific genes (for a detailed review, see [22,23]). *Erk5* deficient mice die around embryonic day (E) 10.5 due to cardiovascular defects and angiogenic failure in embryonic and extra-embryonic tissues. In these mice, the developing vasculature fails to mature, with endothelial cells becoming disorganized and rounded leading to a loss of vascular integrity [24–27]. Similar phenotypic abnormalities are seen in mice lacking *Mek5* [28] and *Mekk3* [29], suggesting that the ERK5 signalling axis is critical to vasculogenesis and angiogenesis. In an attempt to determine the primary defect upon *Erk5* gene ablation, researchers have generated conditional tissue-specific *Erk5*-knockout mice. Endothelial specific *Erk5*-knockout mice show cardiovascular defects and die around E10.0, similar to the conventional *Erk5*-knockout mice [27].However, specific knockout of *Erk5* in cardiomyocytes or hepatocytes does not affect development [27]. Recent studies using an inducible conditional knockout of *Erk5* in neurogenic regions of the mouse brain have shown a role for ERK5 in neurogenesis [30]. Overall, the mouse data suggest that, whereas global *Erk5* knockout affects cardiovascular development, the initial defect occurs in the endothelium and that ERK5 is critical for endothelial cell function. The requirement of ERK5 in the maintenance of vascular integrity is highlighted by the fact that induced ablation of *Erk5* in adult mice is lethal within 2–3 weeks as blood vessels become leaky due to endothelial cell apoptosis [27].

ERK5 and endothelial cell physiology

Inhibition of endothelial apoptosis

As outlined above, targeted deletion of the ERK5 signalling axis in mice suggests that ERK5 plays an essential role in endothelial cell physiology by maintaining endothelial cell survival [22]. The requirement for ERK5 to protect endothelial cells from apoptosis was attributed to the necessity of MEF2C phosphorylation by ERK5 [27]; the phenotype of *Mef2c* −/− mice is similar to that of *Erk5*−/− mice, with embryonic lethality resulting from cardiac and vascular malformations [31–33]. However, it was observed that infection of *Erk5*−/− embryos with an adenovirus encoding a constitutively active Mef2c was only able to partially protect endothelial cells from apoptosis [27], suggesting the existence of effectors downstream of Erk5 that regulate apoptosis [34]. Recent *in vitro* studies using human dermal microvascular endothelial cells (HDMECs) demonstrated that ERK5 is required for vascular endothelial growth factor (VEGF)-mediated phosphorylation of protein kinase B (PKB)/Akt, resulting in increased expression of the pro-survival protein BCL-2 and increased phosphorylation of the pro-

apoptotic protein BAD. This suppresses apoptosis, resulting in increased cell survival facilitating tubular morphogenesis of the endothelial cells in a collagen gel [35] (Figure 2).
A role for ERK5 in regulating PKB/Akt phosphorylation at Ser473 and Thr308 has previously been reported in mouse embryonic fibroblasts under conditions of osmotic stress [36]. In mouse neuronal cells, nerve growth factor (NGF) mediated phosphorylation of PKB/Akt on Ser473, but not Thr308, was dependent on ERK5 activity [37]. Furthermore, ERK5 has also been implicated in platelet-derived growth factor (PDGF)-induced activation of PKB/Akt in porcine aortic endothelial cells (PAEs) [38] and FLT-3-mediated activation of PKB/Akt in the Ba/F3 pro-B-cell line [39]. Taken together, these data suggest that ERK5 may play a critical role in coupling growth factor receptors to activation of PKB/Akt and regulating cell survival; disruption of VEGF mediated PKB/Akt activation in endothelial cells may in part explain the profound vascular phenotype in the *Erk5*−/− mice. This raises the question of how ERK5 mediates PKB/Akt activation. It is unlikely that ERK5 directly phosphorylates PKB/Akt, as it is well established that PKB/Akt is activated via phosphorylation of Thr308 in the activation loop via the mammalian target of rapamycin complex 2 (mTORC2) [40,41], as well as Ser473 in the C-terminus by phosphoinositide-dependent kinase-1 (PDK1) [42]. It may be that ERK5 regulates the activity of a phosphatase, such as protein phosphatase 2 (PP2A) [43] or MKP3 [44], which have been shown to regulate PKB/Akt dephosphorylation. The precise mechanism through which ERK5 regulates PKB/Akt phosphorylation and activation remains obscure.

Shear stress and atheroprotection

Atherosclerosis is a condition in which patchy deposits of fatty material (atheromas or atherosclerotic plaques) develop in the walls of medium-sized and large arteries, leading to reduced or blocked blood flow. Investigations both *in vivo* and *in vitro* have shown that disturbed flow is proatherogenic by promoting oxidative and inflammatory states in the artery wall [45]. In contrast, steady laminar blood flow is atheroprotective by inhibiting oxidative stress and inflammation in the vessel wall [45,46].
Fluid shear-stress-mediated ERK5 activation has been shown to confer an anti-apoptotic effect in bovine lung microvascular endothelial cells (BLMECs) [47] and an atheroprotective effect by negatively regulating tumour necrosis factor *α* (TNF*α*)-stimulated expression of adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and E-selectin in HUVECs [48]. A more recent study utilizing a novel MEK5 inhibitor, BIX02188, has revealed that the MEK5/ERK5 pathway mediates flow-dependent inhibition of TNF*α* signalling in BLMECs [49].
Analysis of laminar shear-stress-induced transcriptional responses in endothelial cells has identified Krüppel-like factor 2 (KLF2) as a mechanostress-induced gene [50,51], which is responsible for negatively regulating inflammation and angiogenesis and maintaining vascular quiescence [51–53]. KLF2 has subsequently been identified as an ERK5-responsive gene in mouse embryonic fibroblasts in a pathway requiring MEF2 transcription factor [54]. In addition, studies have shown that ERK5 is required for flow-induced expression of KLF2 in HUVECs [55] and human glomerular endothelial cells [56]. KLF2 inhibits activation of nuclear factor *κ*B (NF-*κ*B) and subsequently reduces the expression of VCAM-1 and E-selectin on the vascular endothelium, which results in decreased adhesion of leucocytes and an anti-inflammatory response [51]. KLF2 is also implicated in up-regulating endothelial nitric oxide synthase (eNOS) and thrombomodulin expression in endothelial cells, which confer potent anti-thrombotic and anti-inflammatory properties resulting in a vasoprotective effect [51,57]. In addition to KLF2, ERK5 activation induces the expression of KLF4-dependent genes, which are also important in flow-mediated endothelial cell-protective responses [58,59] (Figure 2). Furthermore, statins have been shown to activate KLF4-dependent gene expression viaERK5in endothelial cells, suggesting that some of the pleiotropic vasoprotective effects of these drugs may be elicited through activation of ERK5 [58].
It has recently been observed that the flow-induced activation of ERK5 conveys a cytoprotective effect via activation of nuclear erythroid 2-related factor 2 (NRF2) in HUVECs [60]. NRF2 is a basic region leucine-zipper transcription factor which binds to the antioxidant-response element (ARE) and thereby regulates the expression of a number of genes involved

in the cellular antioxidant and anti-inflammatory defence as well as mitochondrial protection [61] (Figure 2).

Tumour angiogenesis

Angiogenesis is defined as the formation of new blood vessels from pre-existing vessels and plays a critical role both in normal physiological development and in the pathology of diseases such as cancer [62,63]. Hayashi et al. [27] initially reported that both VEGF and fibroblast growth factor 2 (FGF2) stimulate ERK5 activity in HUVECs and mouse lung capillary endothelial cells (MLCECs) [27], raising the possibility that ERK5 regulates angiogenesis in endothelial cells. More recent data have defined a role for ERK5 in regulating VEGF-mediated tubular morphogenesis in HDMECs, but not VEGF-mediated proliferation, in contrast with the role of ERK1/2, which regulates proliferation, but not tubular morphogenesis, in these cells [35]. Hayashi et al. [64] also provided initial evidence that ERK5 regulates tumour angiogenesis. Following the establishment of human melanoma and Lewis lung carcinoma tumour xenografts in mice, induced ablation of *Erk5* in *Erk5*flox/flox mice carrying an inducible *Mx1-Cre* transgene resulted in a regression of the tumour vasculature and a concomitant reduction in tumour volume by 63% and 72% respectively [64]. The recent development of small-molecule inhibitors of MEK5 (BIX02188/BIX02189) [65] and ERK5 (XMD8-92) [66] is now allowing researchers to analyse the therapeutic effect of inhibiting the ERK5 signalling axis using *in vivo* animal models of tumour development. Pharmacological use of XMD8-92 in a mouse human tumour xenograft study revealed that tumour growth was inhibited by 95% and that FGF2-mediated angiogenesis was inhibited in Matrigel plugs [66]. This study also revealed that ERK5 is able to suppress the promyelocytic leukaemia protein (PML) in the nuclear body by direct phosphorylation of Ser403 and Thr409, consequently preventing the up-regulation of p21 expression, an important proliferation modulator [66]. Importantly, this study revealed that no adverse vascular effects were seen with XMD8-92 administration in mice, in contrast with those previously observed in the endothelium-specific deletion of *Erk5* in mice leading to embryonic lethality [27]. This suggests that targeting ERK5 could be a viable anti-cancer therapeutic strategy. Indeed, the data discussed above would suggest that inhibiting ERK5 would simultaneously target both proliferation in cancer cells and VEGF-stimulated tubular morphogenesis in endothelial cells, making this an attractive approach to targeting tumour growth and angiogenesis.

Conclusions and perspectives

It is now becoming apparent that ERK5, the most recently discovered MAPK, plays a critical role in cellular function. Whereas ERK5 appears to be almost ubiquitously expressed in different tissues, the phenotype of the ERK5-knockout mice indicates that it is critical for endothelial cell physiology. However, conditional knockout in other cell types suggests a degree of redundancy with other signalling pathways [22]. *In vivo* and *in vitro* studies have revealed that ERK5 is important for endothelial [23] and neuronal [37] cell survival, suggesting that, under certain conditions, these cell types have a critical dependence on ERK5 activity and may express specific ERK5 substrates not expressed in other cells. The recent development of small-molecule inhibitors of MEK5 and ERK5, combined with *in vitro* and *in vivo* animal models, will allow researchers to determine the roles of the ERK5 signalling axis in normal physiology and pathological conditions such as atherosclerosis and cancer.

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Figure Legends:

Figure 1: The ERK5 signalling axis

ERK5 is activated by a linear signalling cascade. MEKK2/MEKK3 (MAPKKK) phosphorylate MEK5 (MAPKK), which in turn phosphorylates ERK5 (MAPK) on Thr218 and Tyr220 within the activation loop of the kinase domain. The ERK5 protein consists of 816 amino acids and contains a kinase domain, NLS, two proline-rich domains (PR1 and PR2) and a relatively large C-terminal tail of approximately 400 amino acids. Activation of ERK5 facilitates its nuclear localization and phosphorylation of a range of substrates such as MEF2C, c-Myc, Ets domain transcription factor serum-response factor accessory protein 1a (Sap-1), SGK, p90 RSK (reviewed in [21,22]). CSF-1, colony-stimulating factor 1; EGF, endothelial growth factor; LIF, leukaemia-inhibitory factor; NGF, nerve growth factor.

Figure 2: ERK5 and vascular effects

The ERK5 signalling cascade is activated in vascular endothelium in response to flow-induced shear stress and VEGF-A activation of VEGF receptor 2 (VEGFR-2). Blood flow in the normal endothelium results in shear stress due to activation of mechanosensitive channels on the apical surface of endothelial cells. Activation of MEK5/ERK5 results in increased expression of the transcription factors KLF2 and KLF4 which regulate an anti-inflammatory reaction via inhibition of NF-κB. Activation of KLF2 and KLF4 is also known to up-regulate expression of eNOS and thrombomodulin (TM), which results in vasoprotection. Activation of MEK5/ERK5 in response to shear stress also activates NRF2, leading to increased expression of the antioxidant genes haem oxygenase 1 (HO-1) and NAD(P)H dehydrogenase (quinone) 1 (NQO-1). VEGFR-2-mediated activation of MEK5/ERK5 results in phosphorylation of PKB/Akt in endothelial cells, which leads to increased expression of BCL-2 and increased phosphorylation of BAD, resulting in cell survival.

Figure 1

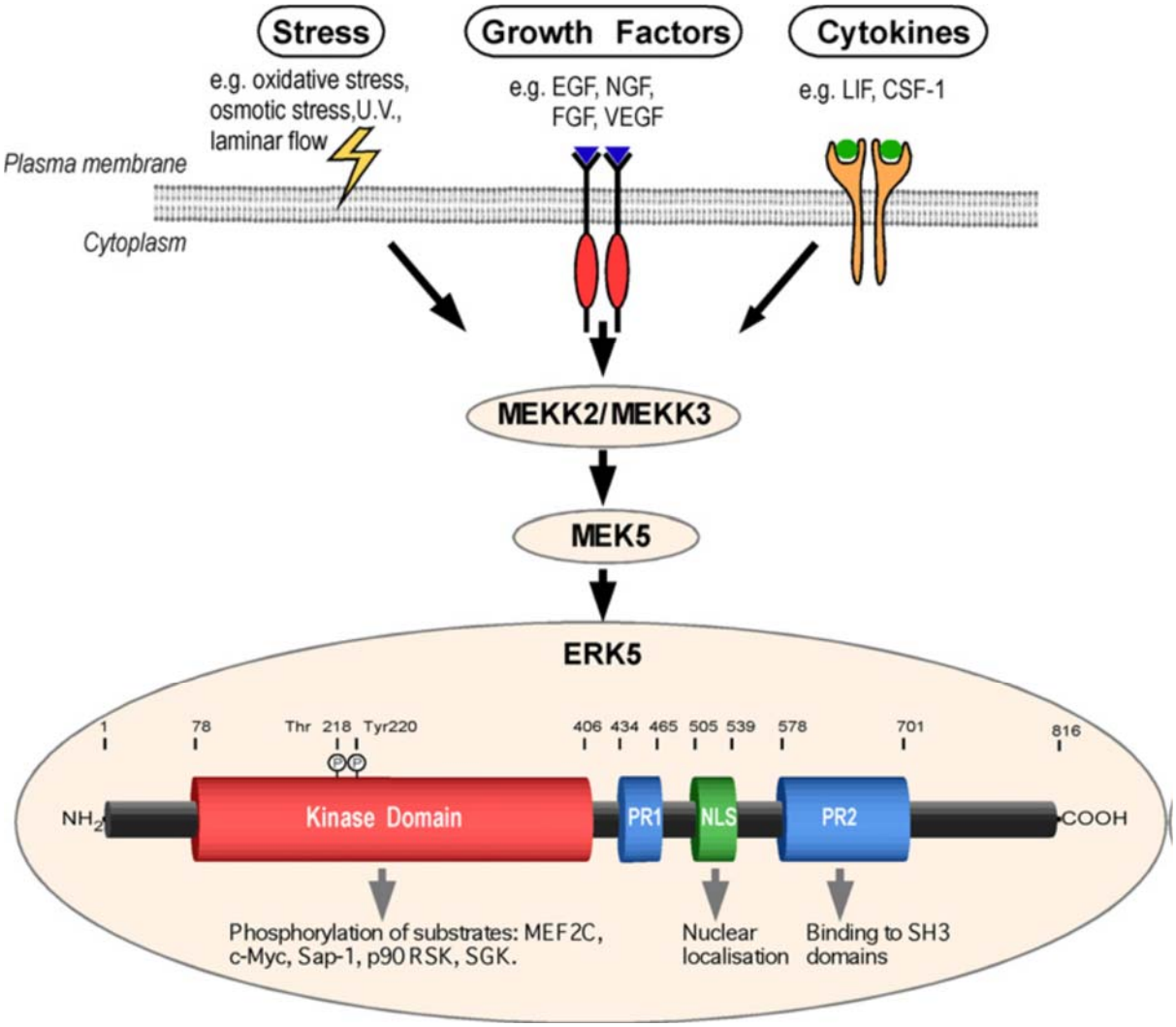
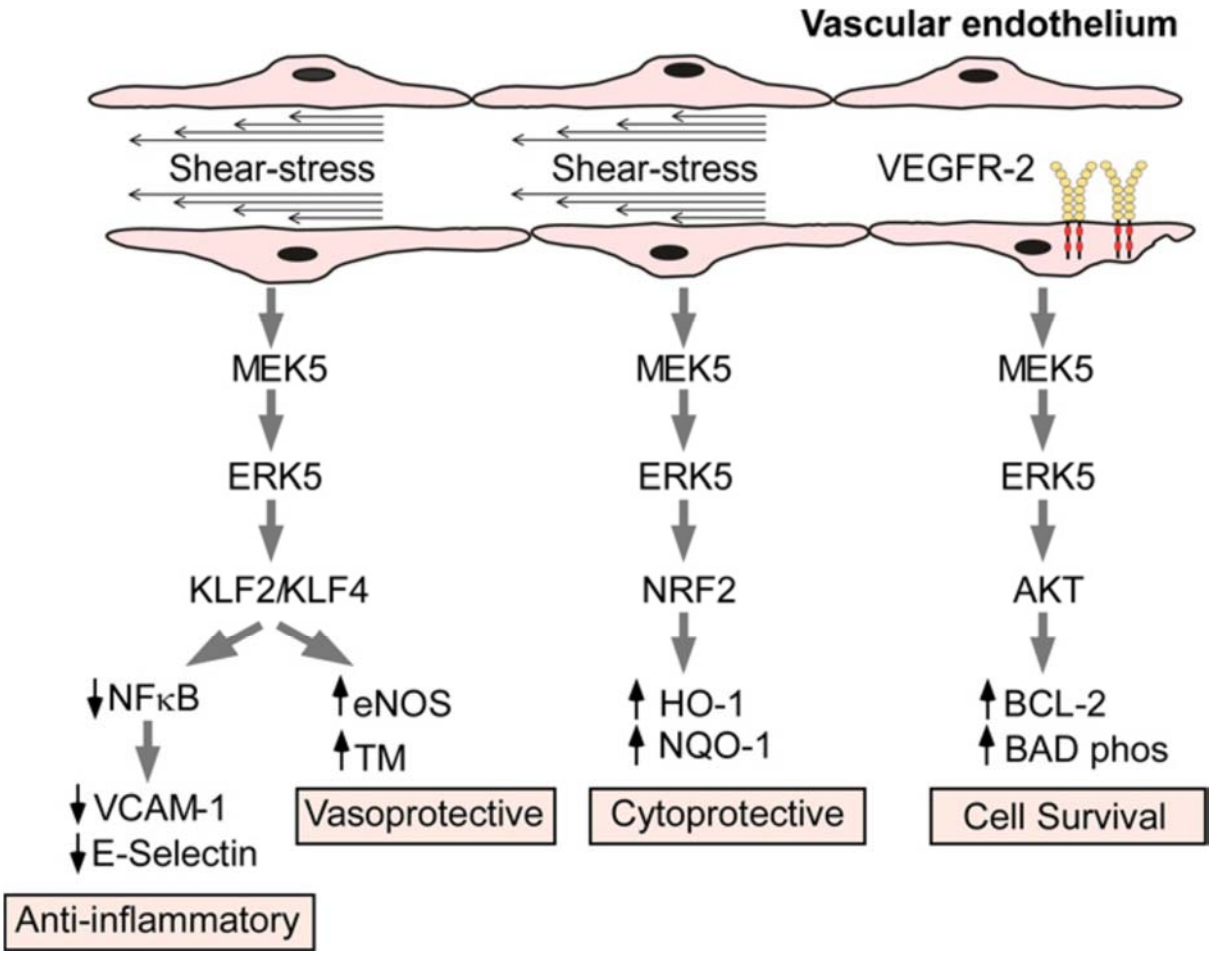

Stress
Growth Factors
Cytokines
e.g. oxidative stress, osmotic stress,U.V., laminar flow
e.g. EGF, NGF, FGF, VEGF
e.g. LIF, CSF-1
Plasma membrane
Cytoplasm
MEKK2/MEKK3
MEK5
ERK5
1
78
Thr 218 Tyr220
406 434 465 505 539
578
701
816
NH2
Kinase Domain
PR1
NLS
PR2
COOH
Phosphorylation of substrates: MEF2C, c-Myc, Sap-1, p90 RSK, SGK.
Nuclear localisation
Binding to SH3 domains

Figure 2


Vascular endothelium
Shear-stress
Shear-stress
VEGFR-2
MEK5
ERK5
KLF2/KLF4
NFκB
VCAM-1
E-Selectin
Anti-inflammatory
eNOS
TM
Vasoprotective
MEK5
ERK5
NRF2
HO-1
NQO-1
Cytoprotective
MEK5
ERK5
AKT
BCL-2
BAD phos
Cell Survival