Cardiovascular Research (2015) **105**, 86–95
doi:10.1093/cvr/cvu236

Erk5 inhibits endothelial migration via KLF2-dependent down-regulation of PAK1

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Received 27 May 2014; revised 9 October 2014; accepted 27 October 2014; online publish-ahead-of-print 11 November 2014

Time for primary review: 23 days

Aims The MEK5/Erk5 pathway mediates beneficial effects of laminar flow, a major physiological factor preventing vascular dysfunction. Forced Erk5 activation induces a protective phenotype in endothelial cell (EC) that is associated with a dramatically decreased migration capacity of those cells. Transcriptional profiling identified the Krüppel-like transcription factors KLF2 and KLF4 as central mediators of Erk5-dependent gene expression. However, their downstream role regarding migration is unclear and relevant secondary effectors remain elusive. Here, we further investigated the mechanism underlying Erk5-dependent migration arrest in ECs.

Methods and results Our experiments reveal KLF2-dependent loss of the pro-migratory Rac/Cdc42 mediator, p21-activated kinase 1 (PAK1), as an important mechanism of Erk5-induced migration inhibition. We show that endothelial Erk5 activation by expression of a constitutively active MEK5 mutant, by statin treatment, or by application of laminar shear stress strongly decreased PAK1 mRNA and protein expression. Knockdown of KLF2 but not of KLF4 prevented Erk5-mediated PAK1 mRNA inhibition, revealing KLF2 as a novel PAK1 repressor in ECs. Importantly, both PAK1 re-expression and KLF2 knockdown restored the migration capacity of Erk5-activated ECs underscoring their functional relevance downstream of Erk5.

Conclusion Our data provide first evidence for existence of a previously unknown Erk5/KLF2/PAK1 axis, which may limit undesired cell migration in unperturbed endothelium and lower its sensitivity for migratory cues that promote vascular diseases including atherosclerosis.

Keywords Endothelial migration • Erk5 • Laminar shear stress • Krüppel-like factor • PAK1

1. Introduction

Endothelial migration is a crucial process involved in embryonic vascular development as well as in vascular regeneration and adaptation in adult organisms. During embryogenesis, both formation of the primitive vascular network (vasculogenesis) and subsequent vessel sprouting (angiogenesis) and remodelling require migration of endothelial cells (ECs).[1] After the onset of the heartbeat, haemodynamic forces shape the final network triggering further remodelling and finally vessel maturation at which stage global endothelial migration activity gradually decreases.[2] Adult vessels then are rather characterized by a state of quiescence than by motility and show enhanced barrier function.[2] Yet, states of hypoxia, inflammation, or vascular damage can awaken the endothelium from its quiescent, immotile state to enable it to adequately respond to environmental cues.[3] This endothelial plasticity is highly desirable in wound healing or as adaptive response to growth and physical training. However, enhanced endothelial migration and angiogenesis is also a hallmark of cancer and contributes to the pathogenesis of vascular diseases including atherosclerosis.[4,5] In the latter, enhanced endothelial migration and angiogenesis, for instance, is closely associated with intraplaque haemorrhage, a proposed risk factor for fatal plaque ruptures.[6] Thus, the elucidation of pathways regulating endothelial migration is of great therapeutic interest.

One important pathway regulating various endothelial functions is the MEK5/Erk5 mitogen-activated protein kinase pathway.[7] It is potently activated by laminar shear stress (LSS) in ECs,[8] a major protective force exerted on the vessel wall by the blood flow that sustains endothelial integrity and function by inducing an anti-apoptotic, anti-thrombotic, and anti-inflammatory gene expression pattern in ECs.[9] Moreover, Erk5 is stimulated by certain vasoprotective drugs such as

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3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins)[10] that, besides their cholesterol-lowering effect, have several other beneficial properties such as angiogenesis modulatory, immunosuppressive, and plaque-stabilizing capacity.[11,12]

Erk5 inhibition was shown to abolish both the anti-apoptotic[13] and anti-inflammatory effects of LSS on ECs [14,15] and also reversed the immunosuppressive action of statins,[16] suggesting that both stimuli mediate at least some of their favourable features via Erk5. In mice, germ line as well as endothelial-specific deletion of Erk5 resulted in embryonic lethality around the onset of blood flow due to various defects in cardiovascular development including increased endothelial apoptosis, vascular leakage, and failure to undergo proper vessel maturation and angiogenesis.[17] The former two defects were also the leading cause of death in adult mice with conditional Erk5 deletion,[18] underscoring a paramount role of Erk5 not only in control of embryonic cardiovascular development but also in maintenance of mature blood vessels.

Consistent with the assumed role of LSS as a physiological regulator of Erk5 activity, we previously demonstrated that expression of a constitutively active variant of its upstream activator MEK5 (MEK5D) in primary human umbilical cord vein ECs (HUVECs) could mimic several of its positive responses and triggered a generally protective gene expression pattern characterized by decreased expression of pro-inflammatory genes as well as induction of vasodilatory, haemostatic, and anti-thrombotic transcripts.[10] Strikingly, MEK5D also significantly regulated various gene clusters associated with endothelial migration and strongly inhibited both migration and angiogenesis *in vitro*.[10,19] The observed migration defect was associated with enhanced cell adhesion, a disturbed actin cytoskeleton and impaired focal adhesion turnover that in part was accounted for by reduced expression of BCAR1/p130 Cas,[19] a known pro-migratory adaptor protein involved in focal adhesion kinase signalling. However, MEK5D-induced BCAR1 repression was only mild and its overexpression could only partially revert the anti-migratory phenotype of MEK5D cells,[19] suggesting that other migration-relevant Erk5 targets are likely to exist.

Our analysis of MEK5D-induced transcriptional changes put the Krüppel-like transcription factors KLF2 and KLF4 into focus as they importantly contributed to Erk5-dependent protective gene expression.[10] Both KLFs represent flow-activated genes and exhibit a strictly LSS-associated gene expression pattern *in vivo* characterized by exclusive expression in LSS-exposed lateral arterial regions, but not in areas exposed to disturbed flow that are prone to atherosclerotic plaque formation.[20,21] Indeed, recent mouse experiments provided evidence for an important atheroprotective role of both factors *in vivo*.[22,23] Forced expression of each of the two KLFs further was shown to inhibit endothelial migration and angiogenesis[10,24,25] implicating a migration-restraining role of KLFs downstream of Erk5. However, their individual role in the anti-migratory response to Erk5 activation remains to be clarified since both factors exhibit a substantial degree of functional and transcriptional redundancy upon overexpression.[10,21] Moreover, crucial migration-relevant secondary targets of Erk5 still await identification.

p21-activated kinases (PAKs) constitute a class of serine/threonine kinases that play important roles in cardiovascular development and endothelial migration.[26,27] The family comprises totally six members subdivided in two classes. Among those, Class I PAKs (covering PAK1–3) are prominent effectors of the small Rho GTPases Rac and Cdc42 that represent central signalling nodules controlling essential processes of cell movement including cell and migration polarity, actin cytoskeleton dynamics, membrane ruffling, filopodium and lamellipodium formation, chemotaxis, and adhesion.[28] Within the family, PAK1 has gained most attention due to its function as oncogene.[29] However, PAK1 is also known for its vessel destabilizing, pro-migratory, and pro-angiogenic function in ECs [30–33] and has recently been implicated with atherosclerotic initiation in mice.[34]

Here, we reveal PAK1 as a novel flow- and statin-responsive target of the MEK5/Erk5 pathway and provide evidence that KLF2-mediated PAK1 repression critically contributes to Erk5-dependent migration arrest in ECs.

2. Methods

2.1 Cell culture and reagents

HUVECs and human primary umbilical artery ECs (HUAECs) were purchased from PromoCell and cultured in a 1 : 2 mixture of endothelial growth medium (EGM, Lonza) and M199 medium (PAA) as described.[10] Cells were used until passage 3–4. For wound healing assays, growth factors and FBS were omitted and instead medium was supplemented with 0.1% bovine serum albumin (BSA, A7030, Sigma). Amphotropic retrovirus producer cells (ϕNXampho) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% foetal bovine serum (PAA). The selective Erk5 inhibitors XMD8-92 (SC-361408) and BIX-02188 (S1530) were purchased from Santa Cruz and Selleck Chemicals.

2.2 Plasmids and retroviral infections

A bicistronic retroviral construct for constitutively active rat MEK5 S311D/T315D (MEK5D) coupling its expression to a GFP/Zeocin resistance fusion gene via an internal ribosomal entry site was described previously.[19] Retroviral constructs for stable expression of wild-type (wt) and kinase-dead (KD) PAK1 (K299R) were constructed by subcloning an *Eco*RI fragment from pCMV6M PAK1 wt and pCMV6M PAK1 KD (K299R)[35] (Addgene) into pBABE puro. Retroviral infection of HUVECs was carried out in two consecutive rounds using 5 μg/mL of polybrene as detailed before.[10] In co-infection experiments, cells were exposed to a 1 : 1 ratio of the respective retroviruses and after 72 h selected for puromycin resistance conferred by the retroviral pBABE puro backbone (2 μg/mL of puromycin, overnight). Cells were then re-seeded into puromycin-free medium for experiments. MEK5D co-expression was routinely confirmed by monitoring GFP positivity.

2.3 Antibodies and western blot

The antibodies used in this study were: Erk5 (Upstate Biotechnology #07-039), MEK5 (Chemicon #AB3184), α-Tubulin (B-5-1-2, Sigma #T5168), PAK1 (Millipore #NG1734260), PAK2 (Cell Signaling #2608), and PAK4 (Cell Signaling #3242). HUVECs were lysed in E1A lysis buffer and proteins were detected by western blot as described.[10]

2.4 Small interfering RNA and transfection

HUVECs (1.2×10^5/well) were seeded into six-well plates and the following day transfected with a pool of two different small interfering RNAs (siRNAs)/target gene (sequences available in Supplementary material online, Information) using oligofectamine (Life Technologies). After a 4-h incubation period, cells were infected with the indicated retroviruses or reseeded for laminar flow experiments. For determination of protein and mRNA expression, lysates were collected 72 h after transfection. Knockdown efficiencies were determined by quantitative real-time PCR (qRT-PCR) or immunoblot.

2.5 Gene expression assays

For gene expression analysis, qRT-PCR was performed by the primer probe method as described[10] using commercially available Taq-Man probes from Life Technologies: GAPDH (hs99999905_m1), KLF2 (hs00360439_g1), KLF4 (hs00358836_m1), and PAK1 (Hs00945621_m1). Test gene expression was each normalized to the expression of GAPDH and related to an experimental control using the comparative threshold cycle method.

2.6 Laminar flow experiments

HUVECs or HUAECs (1.2×10^5) were seeded into 0.4 μm μ-slides (IBIDI) and upon adherence subjected to LSS (20 dyn/cm^2) using a microfluidic slide system (IBIDI). Samples were lysed directly in the μ-slide and analysed for protein or RNA expression.

2.7 Wound healing assays

Wound healing assays were performed as described.[19] Briefly, 48-well plates were coated with fibronectin (20 μg/mL, Millipore) and blocked with 1% BSA. To produce a cell-free space, 1 mm-thick steel inserts were put into the wells and 2×10^5 ECs were seeded onto the unoccupied space. After cell attachment, inserts were removed and migration was monitored by phase contrast microscopy. Images were taken at time points 0 and 18 h after incubation at 37°C, and unoccupied area and cell-covered fractions were determined using the *image J* plug-in *ScratchAssayAnalyzer*[36] as detailed in Supplementary material online, Information.

For experiments with conditioned medium, HUVECs were infected with MEK5D or empty vector and after 72 h incubated with medium lacking growth factors but containing 0.1% BSA. After 24 h, conditioned medium was collected and used undiluted for migration assays.

2.8 Single cell tracking and time-lapse microscopy

About 5000 cells/well were seeded onto a fibronectin-coated eight-well μ-slide (IBIDI) and cells followed for 16 h by multi-position time-lapse microscopy. A minimum of 15 individual cells/well was manually tracked and analysed using freely available plug-ins for *Image J* as detailed in Supplementary material online, Information.

2.9 Statistical analysis

All statistical evaluations were performed using the GraphPad Prism 6 biostatistical software. Details are given in Supplementary material online, Information.

3. Results

3.1 Erk5-mediated inhibition of migration relies on KLF2

Our previous data identified the MEK5/Erk5 pathway as a strong anti-migratory pathway in ECs[19] and established the Krüppel-like factors KLF2 and KLF4 as key transcriptional effectors of active Erk5.[10] Our results implicated KLF4 as a mediator of the Erk5-dependent anti-migratory response since KLF4 expression could partially reproduce the migration and angiogenesis defect induced by Erk5 activation.[10] However, KLF2 overexpression was likewise found to inhibit endothelial migration[25] and angiogenesis.[24] To unequivocally clarify the individual contribution of each KLF to the anti-migratory response to Erk5, we performed *in vitro* migration (wound healing) assays with MEK5D-expressing ECs depleted of KLF2, KLF4, or both KLFs in combination. Surprisingly, only KLF2 knockdown was capable to substantially rescue the wound closure defect of MEK5D-transduced cells. In contrast, knockdown of KLF4 at best had a mild effect and did not antagonize the impaired wound healing of MEK5D cells to a statistically significant extent (*Figure 1A* and *B*). In fact, KLF2 depletion largely restored wound closure of MEK5D cells and no additional pro-migratory effect of combined KLF2/KLF4 knockdown was observed (*Figure 1A* and *B*). qRT-PCR experiments excluded inefficient knockdown of KLF4 to account for this result as its expression was reduced to ~15% of the respective MEK5D-infected scrambled siRNA control upon KLF4 siRNA transfection (see Supplementary material online, *Figure S1A*). Moreover, we could rule out that unequal MEK5D expression was responsible for the differential effect of the two siRNAs since additional immunoblots confirmed equal MEK5D protein expression and Erk5 phosphorylation in the differently transfected siRNA samples (see Supplementary material online, *Figure S1B*). These data suggest that the anti-migratory response to Erk5 is largely due to KLF2 induction, whereas KLF4 plays a minor role.

In light of the critical contribution of KLF2 to the Erk5-induced anti-migratory response, we wondered whether repression of our previously identified migration-relevant Erk5 target, BCAR1,[19] was due to KLF2 induction. Thus, we performed additional siRNA experiments with MEK5D- or vector-infected ECs, respectively. Consistent with previous findings,[19] MEK5D expression in primary human ECs resulted in a slight but significant BCAR1 mRNA reduction to ~80% of its original level (*Figure 1C*). However, neither the depletion of KLF2 nor of KLF4 alone could restore MEK5D-induced BCAR1 expression. Only the combined knockdown of KLF2 and KLF4 successfully normalized BCAR1 mRNA levels, suggesting that BCAR1 represents a common KLF2/KLF4 target (*Figure 1C*). Thus, BCAR1 repression cannot sufficiently account for the strong pro-migratory effect of KLF2 siRNA on MEK5D-infected cells observed in our experiments. We thus reasoned that a yet unknown KLF2 target must be responsible for the robust anti-migratory effect of active Erk5.

3.2 MEK5D represses expression of PAK1

Wound closure assays are excellently suited to detect migration defects of cell populations. Yet, they integrate many factors affecting migration including proliferation, turnover of intracellular contacts, cell substrate interaction or polarized cell motility, and fall short in providing detailed information on migration defects of individual cells. We thus performed single cell tracking experiments of vector- (see Supplementary material online, *Movie S1*) and MEK5D-infected cells (see Supplementary material online, *Movie S2*) to further characterize the migration defect induced by Erk5 activation. *Figure 1D* and Supplementary material online, *Figure S2* illustrate that MEK5D infection of HUVECs strongly affected both the migrated accumulated and Euclidean distances (i.e. the shortest distance between starting and endpoint of migration) as well as their migration velocity and resulted in highly immotile cells (see Supplementary material online, *Movie S2*). This corroborates our earlier finding using electric cell substrate impedance sensing measurements, which revealed substantially decreased micromotions in MEK5D cells and further uncovered aberrancies in focal adhesion distribution as well as the actin cytoskeleton that likely contribute to the migration defect.[19]

Our previous transcriptome analysis of MEK5D-regulated genes in primary human ECs revealed various candidate genes that may contribute to the observed anti-migratory response to Erk5 activation in ECs.[10] Yet, none of those genes could satisfactorily explain the defects in focal adhesion distribution and the actin cytoskeleton observed in MEK5D cells. We thus searched the literature for potential alternative candidates that may have escaped our transcriptome study. An important positive regulator of focal adhesion turnover, actin dynamics,[37] and endothelial migration[32] is the Rac/Cdc42 effector PAK1. Intriguingly, the reported focal adhesion phenotype of PAK-depleted cells was similar to that observed with MEK5D-expressing ECs and likewise characterized by decreased focal adhesion turnover and random focal adhesion distribution.[37] We thus wondered whether MEK5D infection might result in loss of one or multiple PAK isoforms in ECs. Indeed, we found that, compared with vector-infected cells, MEK5D-transduced HUVEC showed a strongly decreased PAK1 mRNA expression and almost

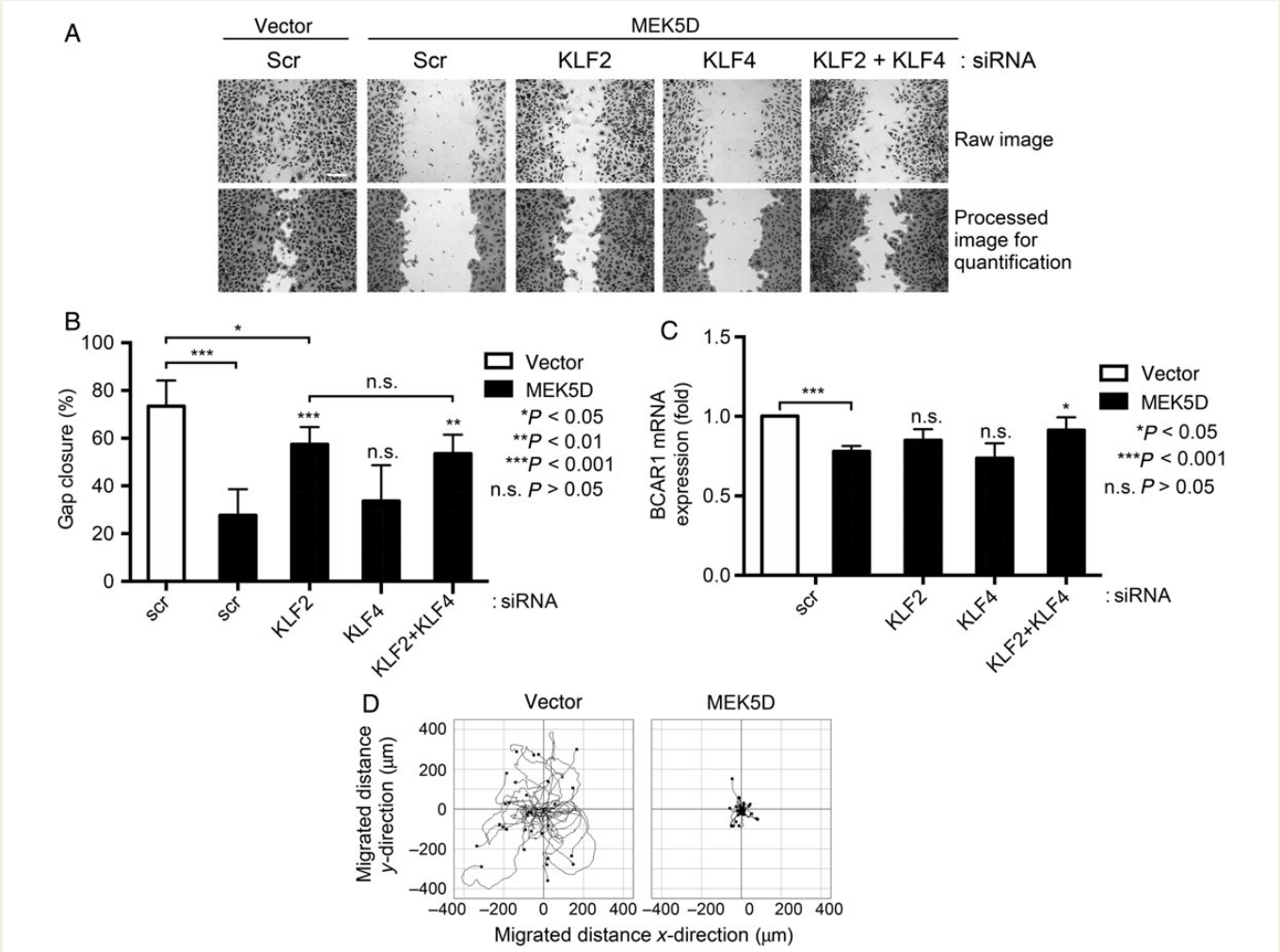

Figure 1 Erk5 inhibits endothelial migration via KLF2. (*A*–*C*) Primary HUVECs were transfected with the indicated siRNAs and after infection with an empty vector or a retrovirus encoding constitutively active MEK5 (MEK5D) either subjected to wound healing assays (*A* and *B*) or processed for BCAR1 mRNA analysis by qRT-PCR (*C*). (*A* and *B*) Knockdown of KLF2, but not KLF4, restores wound healing capacity of MEK5D-transduced ECs. (*A*) Representative microscopic images of $n = 6$ experiments taken 18 h after assay initiation (72 h after siRNA transfection). Lower panels show transparent overlays of raw images and corresponding processed images used for quantification as detailed in Supplementary material online, Information. Scale bar represents 200 μm. (*B*) Quantification of percental gap closure ± SD for the indicated combinations ($n = 6$). (*C*) Effect of KLF2 and/or KLF4 depletion on MEK5D-induced BCAR1 mRNA expression. Data are derived from 4–5 individual experiments and represent mean fold values of GAPDH-normalized mRNA expression ± SD in relation to the vector + si-scrambled (Scr) control (arbitrarily set to 1). (*D*) Representative single cell tracking results of $n = 3$ infection experiments in HUVECs, illustrating the inhibitory effect of MEK5D expression on individual cell trajectories (lines). *XY* diagrams show cell movements in *x*–*y* direction (in μm) with reference to their start position (0) over a period of 16 h. Each dot marks the endpoint of one of $n = 30$ individually tracked cells. In (*B* and *C*), statistically significant changes in comparison with the respective experimental control (Scr + MEK5D) or between the indicated groups (solid lines) were determined by one-way ANOVA followed by Holm–Sidak multiplicity correction. Statistical differences to the normalized vector/scr control in (*C*) were evaluated by one-sample *t*-test followed by Bonferroni–Holm multiplicity correction. Significant multiplicity-adjusted *P*-values are indicated by asterisks.

completely lost PAK1 protein expression (*Figure 2A* and *B*, left). In contrast, no inhibition of PAK2 and PAK4 was observed at both protein and mRNA level (*Figure 2A* and *B*, left) and virtually no mRNA expression of the remaining PAK isoforms, PAK3, 5, and 6, was detected in qRT-PCR experiments (data not shown). Rather similar results were obtained with HUAECs, albeit we detected a slight reduction in PAK2 mRNA that did not alter its protein level (*Figure 2A* and *B*, right). Thus, forced Erk5 activation exerts an isoform-specific inhibitory effect on endothelial PAK1 that is independently of venous or arterial origin of the cells.

Recently, we reported that vasoprotective statins could potently activate MEK5/Erk5 signalling.[10] We thus wondered whether statin treatment could likewise inhibit PAK1 expression. Indeed, simvastatin administration dose-dependently decreased PAK1 mRNA levels in HUVEC (*Figure 2C*). Notably, PAK1 inhibition followed the same concentration-dependency as Erk5 phosphorylation that became apparent at doses between 0.1 and 0.5 μM (*Figure 2C*), implicating that simvastatin-induced PAK1 repression likely relied on Erk5 activation. Consistently, simvastatin treatment also triggered Erk5 activation and PAK1 repression in HUAECs (*Figure 2D* and Supplementary material online, *Figure S3*) and induced mRNA expression of the established Erk5 targets KLF2 and KLF4 both upon MEK5D expression and simvastatin treatment in those cells (*Figure 2A* and Supplementary material online, *Figure S3*).

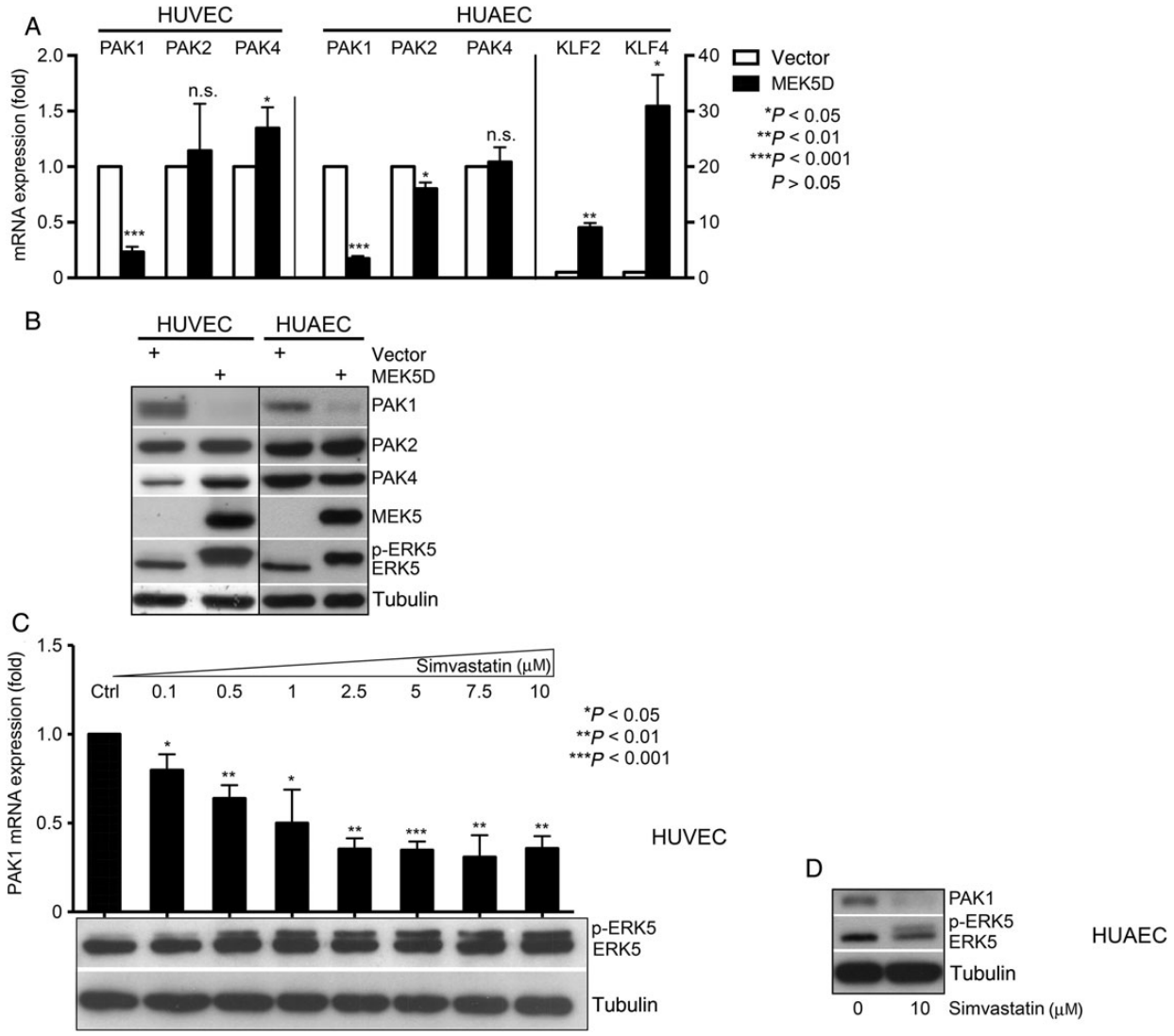

Figure 2 MEK5D expression or statin treatment resulted in decreased PAK1 expression in ECs. (*A* and *B*) HUVECs or HUAECs were infected with the indicated retroviruses and samples were taken 72 h post-infection. (*A*) qRT-PCR experiments showing average fold mRNA expression ± SD of the indicated PAK isoforms or KLF2 and KLF4 in relation to their expression in vector-infected cells (arbitrarily set to 1). Data were calculated from $n = 4$ (HUVEC) or $n = 3$ (HUAEC) experiments upon normalization to GAPDH expression. (*B*) Immunoblots showing isoform-specific repression of PAK1 by MEK5D. Efficient MEK5D expression was monitored by immunoblots for MEK5 and Erk5. Data are representative of $n = 3$ independent experiments. (*C*) qRT-PCR experiments demonstrating dose-dependent reduction of PAK1 mRNA in HUVECs upon 24 h treatment with the indicated simvastatin concentrations. Data are derived from $n = 4$ experiments. To allow comparison to the dose-dependency of simvastatin-induced Erk5 phosphorylation, a representative Erk5 immunoblot is additionally shown. The slower migrating band represents phosphorylated Erk5 (pErk5). (*D*) Western blot confirming simvastatin-dependent PAK1 repression and Erk5 phosphorylation in HUAECs. Tubulin immunoblots in (*B*–*D*) served as a loading control. Statistically significant changes of PAK1 expression in comparison with the respective experimental control [empty vector in (*A*) or diluent control in (*C*)] were determined by one-sample *t*-test. Asterisks indicate statistical significances of calculated uncorrected (*A*) or Bonferroni–Holm multiplicity-adjusted (*C*) *P*-values.

3.3 MEK5D-dependent PAK1 repression is mediated via Erk5 and KLF2

To confirm that the loss of PAK1 expression in MEK5D-transduced cells was mediated via Erk5, we used pharmacological inhibitors for Erk5. Both the highly specific Erk5 inhibitor XMD8-92[38] and the dual-specific MEK5/Erk5 inhibitor BIX02188[39] reverted MEK5D-induced PAK1 repression at concentrations sufficient to block MEK5D-dependent Erk5 phosphorylation (*Figure 3A* and Supplementary material online, *Figure S4*), indicating that the observed PAK1 inhibition was not due to a potential unspecific action of the active MEK5D mutant.

To further analyse whether KLF2 induction was responsible for the observed PAK1 repression by MEK5D, we employed siRNA. *Figure 3B* and *C* illustrates that knockdown of KLF2 was sufficient to normalize PAK1 mRNA and protein levels in MEK5D-infected HUVEC, whereas transfection with KLF4 siRNA had no effect on MEK5D-induced PAK1 repression. Consistent with the expendability of KLF4 for MEK5D-mediated PAK1 repression, PAK1 mRNA levels in MEK5D-infected ECs co-transfected with KLF2/KLF4 siRNAs were statistically indistinguishable from siKLF2-transfected MEK5D-positive cells (*Figure 3B*). Overall, these data implicate KLF2 as a specific, non-redundant PAK1 repressor in ECs.

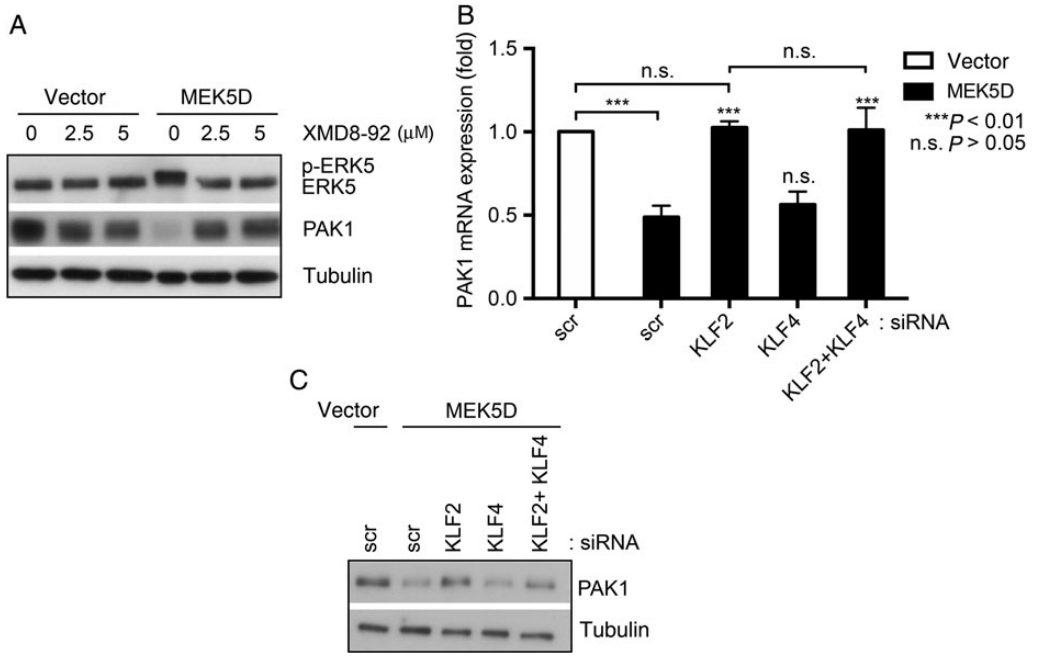

Figure 3 MEK5D-dependent PAK1 repression is mediated by Erk5 and KLF2. (*A*) Western blot showing normalization of MEK5D-induced PAK1 protein expression upon incubation with the indicated concentrations of the specific Erk5 inhibitor XMD8-92 for 72 h. A blot for Erk5 is shown to confirm efficiency of Erk5 inhibition. Tubulin served as the loading control. Data are representative for two independent experiments. (*B* and *C*) Reversion of MEK5D-dependent PAK1 mRNA and protein repression by KLF2 siRNA. HUVECs were transfected with the indicated siRNAs and upon infection with the indicated retroviruses analysed for PAK1 mRNA (*B*) or protein expression (*C*) by qRT-PCR or immunoblot, respectively. Data in (*B*) are derived from $n = 4-5$ experiments and represent mean fold values of GAPDH-normalized PAK1 mRNA expression $\pm$ SD in relation to the vector + Scr control (set to 1). Differences relative to the Scr + MEK5D control or between indicated samples were statistically evaluated by one-way ANOVA or one-sample *t*-test followed by Holm–Sidak/Holm–Bonferroni multiplicity correction, respectively. Statistically different multiplicity-adjusted *P*-values are indicated by asterisks. Blots in (*C*) are representative of $n = 3$ independent experiments.

3.4 Laminar flow represses PAK1 via KLF2

To confirm our findings in a more physiological setting, we performed LSS experiments. In agreement with published data,[8] exposure of HUVEC to LSS resulted in a robust activation of Erk5 as indicated by electromobility shift to its slower migrating phosphorylated form (*Figure 4A*). Erk5 phosphorylation correlated with a time-dependent increase of KLF2 and KLF4 mRNA (*Figure 4B*, left) as well as an appropriately timed decrease of PAK1 at both the protein and mRNA level (*Figure 4A* and *B*, right). Similar results were obtained with HUAEC (*Figure 4C*), excluding a vein-specific effect of these findings. Subsequent siRNA experiments confirmed a dependency of flow-mediated PAK1 repression on KLF2 since PAK1 mRNA expression was preserved in KLF2 siRNA-transfected HUVEC upon flow treatment, but was only slightly increased upon KLF4 siRNA transfection under these conditions (*Figure 4D*).

3.5 PAK1 re-expression increases migration of MEK5D-infected ECs

The best-established endothelial function of PAK1 is its pro-migratory action.[32] To evaluate whether PAK1 loss thus could account for the observed migration defect of MEK5D cells, we performed reconstitution experiments in MEK5D-expressing ECs. Indeed, single cell tracking experiments in HUAECs showed that re-expression of wt PAK1 (PAK1 WT) partially reverted the migration defect of MEK5D-infected cells (*Figure 5A*). In particular, we observed a statistically significant improvement of migrated Euclidean distances upon PAK1 WT co-expression (see Supplementary material online, *Figure S5A*). This was especially evident using threshold analysis, which demonstrated a consistently high ratio of PAK1 WT/MEK5D co-expressing cells migrating >100 μm (corresponding to $\sim 2\times$ the mean Euclidean distance migrated by the MEK5D/vector combination) away from their starting position, whereas MEK5D/vector co-infected cells hardly ever moved beyond this range (*Figure 5A*). Unlike Euclidean distance, mean accumulated distances and mean velocity of MEK5D/PAK1 WT co-expressing cells did not significantly differ from the MEK5D/vector combination (see Supplementary material online, *Figure S5B* and *C*). Yet, threshold analysis revealed that substantially more MEK5D/PAK1 WT co-expressing cells moved at velocities faster than 15 μm/h (equivalent to $\sim 1.5\times$ the velocity determined for the vector/MEK5D combination; *Figure 5A*). Most strikingly, however, we observed a distinct defect in directional movement for MEK5D-/vector-co-infected cells that was fully restored upon PAK1 WT re-expression (*Figure 5B*). Thus, the observed PAK1 loss in MEK5D-expressing cells primarily appears to affect directional movement.

To analyse to which extent these results are transferable to migration of cell populations, we additionally employed wound healing assays. *Figure 5C* and *D* illustrates that PAK1 WT co-expression significantly increased gap closure in MEK5D-expressing HUVECs. In contrast, co-expression of KD PAK1 (PAK1 KD) was insufficient to promote wound healing of MEK5D cells (*Figure 5D*), suggesting a requirement of PAK1 kinase activity. This was also evident from single cell tracking

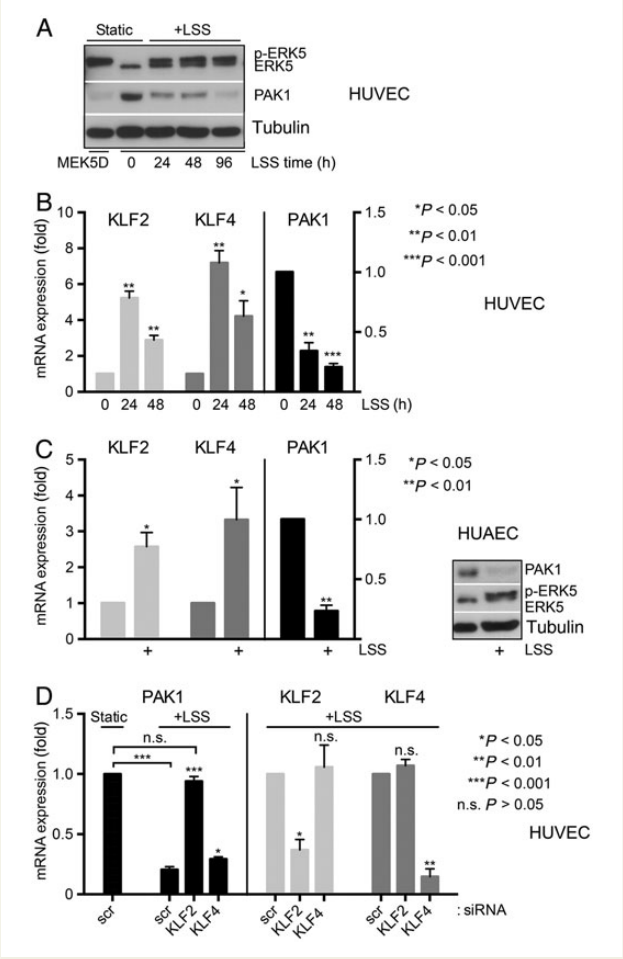

Figure 4 Flow-induced PAK1 repression depends on KLF2, but not on KLF4. (*A*–*C*) LSS represses PAK1. HUVECs (*A* and *B*) or HUAECs (*C*) were left untreated or exposed to LSS (20 dyn/cm^2) for 72 h or the indicated periods of time. Subsequently, cells were harvested and analysed for Erk5 phosphorylation and PAK1 protein expression by immunoblot (*A* and *C*, right) or mRNA expression of KLF2, KLF4, and PAK1 by qRT-PCR (*B* and *C*, left), respectively. In (*A*), a lysate from MEK5D-transduced ECs was included as a positive control for comparison. Immunoblots for tubulin served as a loading control. (*D*) qRT-PCR analysis demonstrating reversion of LSS-induced PAK1 mRNA repression upon transfection with KLF2 siRNA. Immunoblots in (*A* and *C*) are representative of $n = 3$ similar experiments. mRNA data in (*B*–*D*) are derived from $n = 3$ experiments and represent GAPDH-normalized mean fold mRNA expression $\pm$ SD of the indicated genes in relation to the respective static or LSS-treated control (set to 1). Statistical evaluation was performed by one-way ANOVA followed by Holm–Sidak multiplicity correction (*D*, left, comparisons between the LSS/siRNA-co-treated groups and the LSS/scr control) or one-sample *t*-test (all others). Statistical differences according to uncorrected or multiplicity-adjusted *P*-values are marked by asterisks.

experiments since only PAK1 WT but not PAK1 KD re-expression improved motility of MEK5D-infected HUVECs (see Supplementary material online, *Figure S6*).

Of note, in none of our readouts, PAK1 WT re-expression was sufficient to completely normalize migration in MEK5D cells. While down-regulation of BCAR1 may, in part, explain the incomplete rescue observed upon PAK1 WT re-expression, the pro-migratory effect of KLF2 siRNA was still more prominent (compare *Figure 1B* and *5D*). Although we cannot exclude that inappropriately high PAK1 levels might also inhibit migration as previously proposed,[32] we did not observe significantly altered gap closure of vector-transduced ECs upon PAK1 WT co-infection (*Figure 5D*). Immunoblotting further confirmed that MEK5D and PAK1 were co-expressed in our experiments (see Supplementary material online, *Figure S7*). Thus, it is likely that still other KLF2 targets might contribute to the anti-migratory response to Erk5 activation. This may include soluble factors such as the anti-migratory protein, SEMA3F, which represents a known KLF2-induced gene[25] and has likewise been identified as a MEK5D target in our previous microarray.[10] To analyse whether a paracrine mechanism may contribute to the decreased migration of MEK5D-infected ECs, we compared the migration capacity of vector- or MEK5D-infected cells with that of uninfected HUVECs treated with supernatants from MEK5D- or vector-infected cells, respectively. However, no significant changes in gap closure were observed when untransfected HUVECs were incubated with supernatant from MEK5D-expressing cells (see Supplementary material online, *Figure S8*). Thus, the induction of released factors such as SEMA3F is unlikely to substantially contribute to the anti-migratory effect of MEK5D.

4. Discussion

Our data for the first time reveal PAK1 as flow- and statin-repressed genes and migration-relevant target of the MEK5/Erk5/KLF2 pathway (*Figure 6*). Based on the established preventive role of LSS and statins in atherosclerosis, Erk5 and its transcriptional mediators KLF2 and KLF4 have long been proposed to act as atheroprotective factors, a notion supported by recent knockout studies.[22,23,40] It is thus tempting to speculate that the observed inhibitory effect of Erk5/KLF2 signalling on migration and PAK1 expression may likewise serve a protective purpose and functions as a natural mechanism to limit pathological migration in LSS-exposed endothelium. Intriguingly, enhanced endothelial migration and angiogenesis is a common event during advanced atherosclerosis where it correlates with intraplaque haemorrhage and atherosclerotic plaque instability,[6] a condition antagonized by statins.[12] Although the role of PAK1 in this context remains to be elucidated, PAK1 deficiency has recently been shown to suppress atherosclerosis development implicating a role of PAK1 in atherosclerotic initiation.[34] Interestingly, PAK1 phosphorylation also has been reported to exclusively occur at atherosclerosis-prone arterial areas such as branch points and bends, which are characterized by disturbed flow, whereas lateral arterial sites that typically are exposed to atheroprotective laminar flow were devoid of PAK1 activity.[41] Although total PAK1 expression has not been analysed in this previous study, our discovery that PAK1 represents a flow-regulated repression target of KLF2 implies that the lack of PAK1 phosphorylation at the lateral arterial segments may result from missing PAK1 expression since these areas are also characterized by high KLF2 expression.[20] Remarkably, PAK1 has not only been implicated with endothelial migration but also with vascular inflammation[34,42] and regulation of vascular permeability,[30,31] in particular during atherogenesis.[41] All these functions are counteracted by the MEK5/Erk5/KLF2 pathway,[10,18,25,43–46] suggesting that the consequences of the observed loss of PAK1 expression might be more far-reaching and contribute to several other protective functions of this pathway.

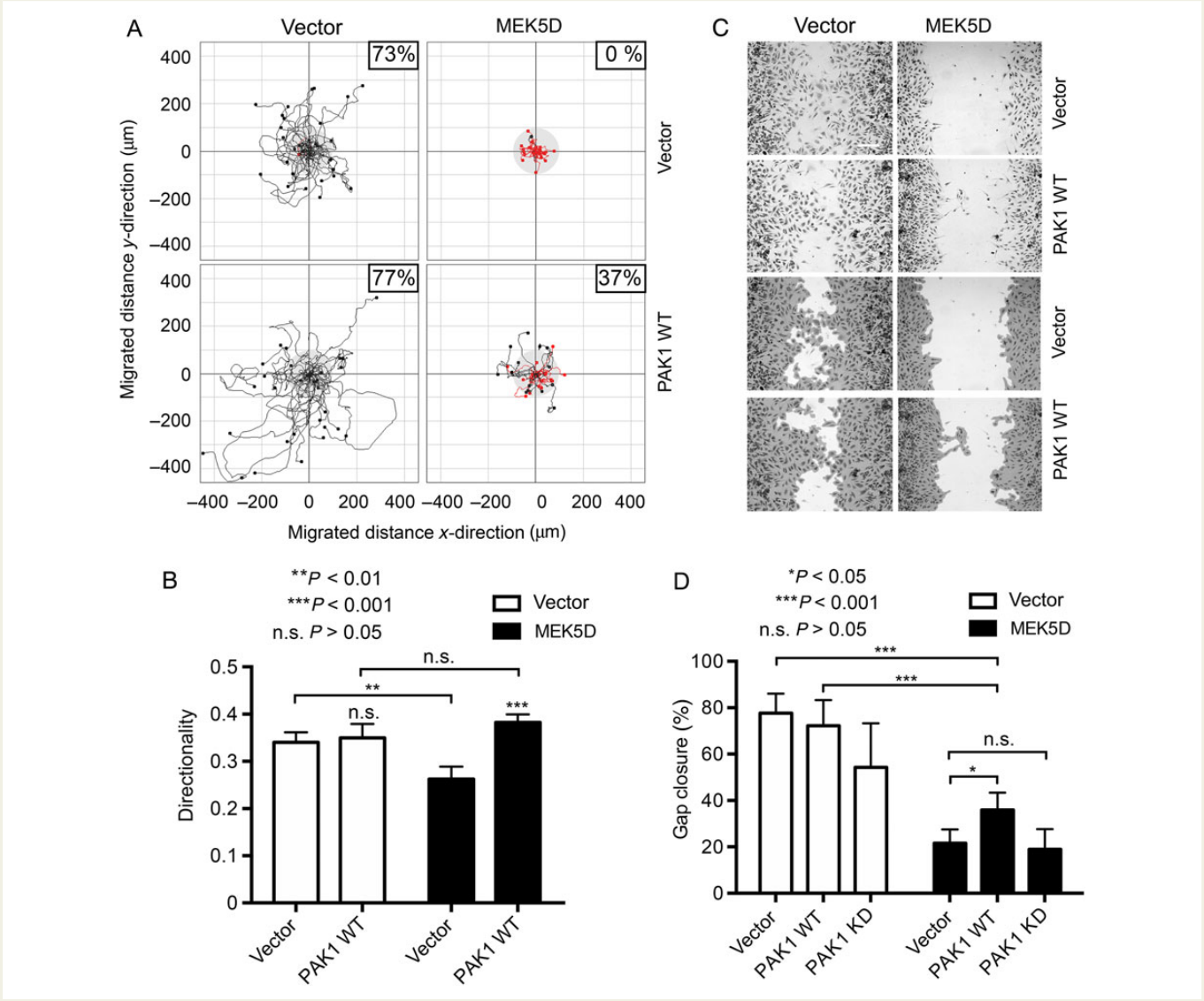

Figure 5 PAK1 re-expression partially restores migration capacity of MEK5D-infected ECs. Primary HUAECs (*A* and *B*) or HUVECs (*C* and *D*) were co-infected with the indicated combinations of empty vector/MEK5D together with PAK1 WT or PAK1 KD and subjected to single cell tracking (*A* and *B*) or wound healing assays (*C* and *D*). (*A*) *XY* diagram showing individual cell trajectories of $n = 30$ cells per indicated group. Data are representative of $n = 4$ independent tracking experiments with at least $n = 15$ cells per manipulation. Percentages of cells migrating Euclidean distances >100 µm (grey-shaded area) are indicated in the upper right corner of each XY diagram. Trajectories of cells slower than 15 µm/h are indicated in red, and those with faster velocity in black with dots in the respective colour marking the migration endpoints after 16 h. (*B*) Bar diagram, illustrating loss of directionality upon MEK5D expression and complete restoration upon PAK1 WT co-infection. Bars show mean directionality (defined as averaged ratio between migrated Euclidean and accumulated distances with a maximal value of 1 being a straight line) ± SD derived from $n = 4$ independent tracking experiments with a minimum of 15 manually tracked cells per indicated co-infection group. (*C*) Representative microscopic images of $n = 8$ experiments taken 18 h after assay initiation. Lower panels depict transparent overlays of unprocessed images (top) and finally processed segmented images used for assay quantification. Scale bar represents 200 µm. (*D*) Quantification of mean percentile gap closure ± SD from $n = 6–8$ independent experiments. Data in (*B* and *C*) were statistically evaluated by two-way ANOVA followed by Holm–Sidak multiplicity testing. Asterisks indicate statistical differences between indicated groups based on determined multiplicity-adjusted *P*-values.

Our observation that KLF2 siRNA largely restored the migration defect of MEK5D cells suggests that KLF2, but not KLF4, mediates the robust anti-migratory function of Erk5 activation. This is consistent with earlier observations that KLF2 expression inhibits both endothelial migration[25] and angiogenesis,[24] but contradicts our previous finding that KLF4 overexpression likewise repressed endothelial migration.[10] However, others and we previously observed that forced KLF4 expression could also modulate expression of normally KLF2-restricted genes.[10,21] For instance, we previously noted that KLF4 overexpression could induce the expression of the anti-thrombotic gene thrombomodulin, while siRNA experiments in MEK5D-infected ECs revealed thrombomodulin as an exclusive KLF2 target.[10] Thus, in an overexpression setting, both KLFs may act partially redundant. Yet, the example of BCAR1 provided in this study emphasizes that both factors also co-regulate several functional genes as previously noted.[10] This view is also supported by a recent *in vivo* study providing evidence that both

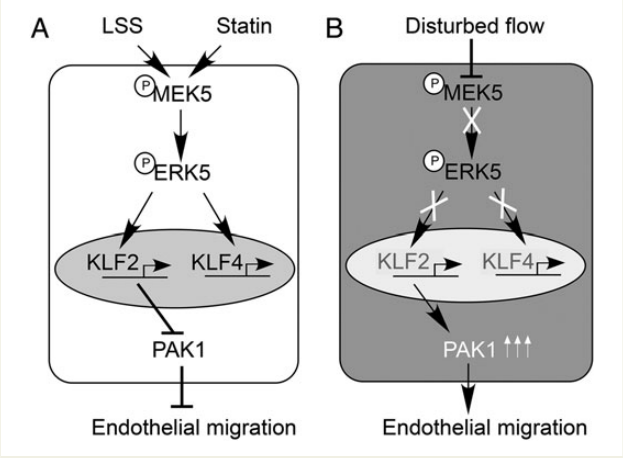

Figure 6 Model of Erk5-dependent migration control in ECs. (*A*) Stimulation of Erk5 activity by LSS leads to induction of KLF2 and subsequent loss of PAK1 expression, which limits migratory processes in healthy endothelium. (*B*) Flow dysfunction triggers loss of Erk5 activity and KLF2 expression, resulting in increased PAK1 levels and enhanced endothelial susceptibility for pro-migratory cues. Statin treatment can reactivate the Erk5/KLF2/PAK1 axis under such conditions restoring the anti-migratory phenotype of ECs (*A*). PAK1 regulation thus may serve as flow-sensitive switch to adjust endothelial susceptibility to pro-migratory cues, suggesting that interference with PAK1 signalling may be a promising strategy to suppress pathological migration in the context of vascular diseases such as atherosclerosis.

genes are required to maintain endothelial integrity in mouse embryonic vascular development.[47] Although our data clearly implicate a major function of KLF2 regarding the regulation of anti-migratory effects of Erk5, other responses may likewise be regulated by KLF4 or both KLFs in concert. Future experiments should address this important issue.

In summary, our data demonstrate that PAK1 loss importantly contributes to the anti-migratory effect of the Erk5/KLF2 module and highlight PAK1 down-regulation as a key mechanism by which Erk5 limits migratory processes in intact endothelium.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online

Acknowledgements

We thank Helga Sennefelder, Silke Garkisch, and Nicole Schmidt for excellent technical assistance and Johannes Hain, Department of Statistics (Institute of Mathematics), University of Würzburg, for help with the statistical evaluation of our data. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Conflict of interest: none declared.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft ('International Research Training Group Vascular Medicine', Graduiertenkolleg 880/3 to M.S. and M.G.) and from the Interdisciplinary Centre for Clinical Research (IZKF) Würzburg (Z-4/116 to M.S.).

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