Nesprin-1 and Nesprin-2 Regulate Endothelial Cell Shape and Migration

Samantha J. King,1,2 Karolin Nowak,1,3 Narendra Suryavanshi,1 Ian Holt,4 Catherine M. Shanahan,2 and Anne J. Ridley1#

1Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt’s House, Guy’s Campus, London, SE1 1UL, United Kingdom
2Cardiovascular Division and British Heart Foundation Centre of Research Excellence, King’s College London, James Black Centre, Denmark Hill Campus, London SE5 9NU, United Kingdom
3University College London Master’s Programme in Molecular Medicine, Division of Infection and Immunity, Gower Street, London WC1E 6BT, United Kingdom
4Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, Shropshire SY10 7AG, United Kingdom

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Nesprins are large multi-domain proteins that link the nuclear envelope to the cytoskeleton and nucleoskeleton. Here we show that nesprin-1 and nesprin-2 play important roles in regulating cell shape and migration in endothelial cells. Nesprin-1 or nesprin-2 depletion by RNAi increased endothelial cell spread area and the length of cellular protrusions, as well as stimulating stress fibre assembly which correlated with an increase in F-actin levels. Nuclear area was also increased by nesprin depletion, and localization of the inner nuclear membrane protein emerin to the nuclear envelope was reduced. Depletion of nesprin-1 or nesprin-2 reduced migration of endothelial cells into a cell-free area, and decreased loop formation in an in vitro angiogenesis assay. Taken together, our results indicate that nesprin-1 and nesprin-2 both regulate nuclear and cytoplasmic architecture, which we propose leads to their effects on endothelial cell migration and angiogenic loop formation.

Key Words: nuclear envelope; endothelial cells; actin cytoskeleton; angiogenesis; cell morphology; cell migration

Introduction

Cell migration is essential for the development of multicellular organisms, as well as wound healing and immune responses. Cell migration also contributes to disease progression, including cardiovascular diseases such as atherosclerosis, and cancer [Chi and Melendez, 2007; Friedl and Alexander, 2011]. The process of cell migration therefore needs to be tightly regulated. The actin cytoskeleton and actomyosin contractility provides the forces necessary for cell migration [Ridley, 2011]. In order for the nucleus to move in concert with the cell membrane and body, the nucleus needs to be associated with the actin cytoskeleton [Gundersen and Worman, 2013].

Nesprins play an important role in linking the nucleus to the cytoskeleton and are key components of the linker of the nucleoskeleton and cytoskeleton (LINC) complex; which transduces mechanical signals from the cytoskeleton to the nuclear lamina [Lombardi et al., 2011; Razafsky and Hodzic, 2009]. There are four nesprin-encoding genes in humans, SYNE1–4, which encode nesprins 1–4. By interacting with the Sad1p-UNC84 (SUN) domain proteins SUN1 and SUN2 in the inter-membrane space, the conserved C-terminal KASH domains of nuclear membrane-targeted nesprins link the nucleus to actin filaments (nesprins 1 and 2), intermediate filaments (nesprin-3) and microtubules (nesprin-4) [Crisp et al., 2006; Mellad et al., 2011; Sosa et al., 2012]. SUN proteins have transmembrane domains across the inner nuclear membrane, and in turn interact with lamin A. Lamin A is a nuclear intermediate filament protein that forms the nucleoskeleton associated with the inner side of the inner nuclear membrane [Mejat and Misteli, 2010]. Thus nesprins, together with SUN proteins and lamin A comprise the LINC complex, resulting in decreased mechanical tension in cells and nuclear deformation [Lombardi et al., 2011; Mellad et al., 2011].

Full-length nesprin-1 and nesprin-2 giant isoforms (nesprin-1 ~ 1 MDa in size and nesprin-2 ~800 kDa) are...
the two largest proteins in the human genome after the protein titin (~3.8 MDa). They are multi-domain proteins with a paired calponin-homology domain (CH) at the N-terminus that binds to actin filaments, and a C-terminal KASH domain that traverses the outer nuclear membrane and interacts with the C-termini of SUN proteins in the inter-membrane space. The central rod domain of nesprins is composed of multiple spectrin repeats [Autore et al., 2013; Mellad et al., 2011]. Each nesprin gene has multiple splice variants, generating a wide range of different protein isoforms. Smaller N-terminally truncated isoforms lacking the CH domains localize to the inner nuclear membrane and interact with lamin A/C and emerin while several nesprin-1 and nesprin-2 isoforms that lack the KASH domain have been shown to localize to other regions of cells, including focal adhesions [Rajgor et al., 2012]. Both nesprin-1 and nesprin-2 are widely expressed however the expression of different isoforms varies between tissues [Rajgor et al., 2012; Zhang et al., 2001].

Mutations in the LINC complex proteins nesprins, lamins and emerin have been implicated in laminopathic diseases associated with cardiac and skeletal muscle defects including Emery Dreifuss Muscular Dystrophy and Dilated Cardiomyopathy [Capell and Collins, 2006; Maraldi et al., 2011; Zhang et al., 2007a]. Nesprin-1 mutants and SUN1/2 mutants in mice exhibit defects in nuclear positioning in skeletal muscle, correlating with reduced exercise capacity [Lei et al., 2009; Zhang et al., 2010] while nespin-1/2 KASH double knockouts show skeletal muscle defects and die at birth due to failed respiration [Zhang et al., 2007b]. It is thought that muscle tissues in vivo are particularly affected by disruption of the LINC complex due to the mechanical forces associated with cellular contraction. However, nesprins are widely expressed and the LINC complex is important in other cell and tissue types, particularly during cellular processes such as migration where mechanical coupling of the cell to the nucleus is also essential [Gundersen and Worman, 2013]. Indeed, mice with both nesprin and SUN protein depletion show severe neuronal migration defects [Zhang et al., 2009], and nesprins are important for nuclear positioning during cell migration in several cell types including fibroblasts and endothelial cells [Chancellor et al., 2010; Luxton et al., 2010].

Endothelial cells (ECs) line blood vessels, and provide a regulatable barrier between the blood and tissues. ECs initiate the formation of new blood vessels through angiogenic signalling. Angiogenesis is important during mammalian development to allow all tissues to have an adequate blood supply [Marcelo et al., 2013]. ECs respond to multiple pro-angiogenic stimuli, such as VEGF and FGF, both in vitro and in vivo [Arroyo and Iruela-Arispe, 2010]. Angiogenesis is known to contribute to the progression of atherosclerosis; in particular, atherosclerotic plaques containing blood vessels are more prone to rupture, which promotes disease progression [Ho-Tin-Noe and Michel, 2011]. In cancer, angiogenesis is central to the growth and metastasis of solid tumours [Carmeliet and Jain, 2011] A clearer understanding of the mechanisms underlying EC migration and angiogenesis will therefore increase our understanding of diseases such as atherosclerosis and cancer, and in the long term could contribute to the development of new therapies.

Here, we investigate the roles of nesprin-1 and nesprin-2 in ECs, focusing on their roles in regulating cell morphology, actin cytoskeletal organization, and cell migration. We find that nesprin-1 or nesprin-2 depletion increases filamentous actin (F-actin) in cells, reduces migration and decreases angiogenesis in a loop formation assay.

Results

Nesprin-1 and Nesprin-2 Expression in Endothelial Cells

To test the roles of nesprin-1 and nesprin-2 in ECs, we first investigated which of the major isoforms were expressed (Supporting Information Fig. 1) [Duong et al., 2014]. Quantitative PCR (qPCR) analysis indicated that, of the isoforms investigated, the most abundant isoform was nesprin-2 giant, however the combination of nesprin-1 isoforms most highly expressed in HUVECs (nesprin-1 giant, nesprin-1B1, nesprin-1α2) is similar to the amount of nesprin-2 giant isoform (Fig. 1a and Supporting Information Fig. 2a).

We used RNAi to deplete nesprin-1 and nesprin-2 expression in HUVECs (Supporting Information Table 1). Four different siRNAs for nesprin-1 and five different siRNAs for nesprin-2 were used to ensure reproducibility and to exclude off-target effects of any one siRNA. The siRNAs were designed to target different exons that are all contained within the giant isoforms as well as the major C-terminal isoforms expressed in HUVECs (nesprin-1/2 giant, 1B1, 1α2) which have been shown to be important in maintaining the integrity of the LINC complex [Zhang et al., 2007a] (Supporting Information Fig. 1). It was not possible to test reproducibly for protein knockdown due to multiple bands detected with nesprin antibodies in HUVECs (data not shown). Therefore, qPCR was used to measure the levels of nesprin mRNAs. As nesprin-1 and nesprin-2 have many splice variants, qPCR was carried out with oligonucleotides specific for the exons targeted by each siRNA to confirm knockdown (Supporting Information Table 2). Each significantly reduced mRNA levels for its relevant exon (Fig. 1b). In contrast to the effects of nesprin-1 and nesprin-2 siRNAs on their targeted exons, they did not reduce the levels of the respective nesprin-1 or nesprin-2 KASH domain exons (Supporting Information Fig. 2b). This could reflect the existence of isoforms in addition to those already characterized (Supporting Information Fig. 1), or compensation in KASH domain expression after nesprin knockdown as previously described [Rajgor et al., 2012]. In addition, nesprin-1 siRNAs did not affect...
nesprin-2 exons tested, and vice versa (Supporting Information Fig. 2b and data not shown). Therefore, results from this study refer specifically to the nesprin regions depleted and cannot be extrapolated to all isoforms.

**Nesprin-1 and Nesprin-2 Affect Endothelial Cell Morphology**

Nesprin-1 and nesprin-2 siRNAs altered HUVEC spread area and F-actin distribution (Fig. 2a). Cells had an increase in stress fibres traversing the cell centre compared to cells transfected with control non-targeting siRNA (Fig. 2a and 2b). Consistent with this, the mean F-actin levels/pixel (pixel intensity) in nesprin-depleted cells were higher than in control cells (Fig. 2c and Supporting Information Fig. 2c). Stress fibres usually terminate in focal adhesions, except in confluent ECs where they frequently end in adherens junctions [Millan et al., 2010]. We therefore investigated whether nesprins affected focal adhesions. Localization of focal adhesions as visualized by paxillin staining (Fig. 3a) or zyxin staining (data not shown) was not altered by nesprin siRNAs; additionally levels of the focal adhesion proteins paxillin, vinculin and zyxin did not change upon nesprin depletion (Supporting Information Fig. 3a; data not shown). Therefore, these regions of nesprin-1 or nesprin-2 affect stress fibres without visibly altering focal adhesions.

The spread area of nesprin-depleted cells was increased compared to control cells (Figs. 2a and 3b and Supporting Information Fig. 3b). Spread area did not correlate with F-actin levels/pixel (data not shown), and thus the increase in F-actin/pixel was not caused by altered cell shape. Nesprin-1 and nesprin-2 knockdown also increased nuclear spread area (Fig. 3c and Supporting Information Fig. 4a). This is in accord with observations that nesprin-2 mutants affect nuclear area [Lu et al., 2012].

These results were all observed with the majority of siRNAs, indicating that they are unlikely to be due to reduction in only one splice variant of each gene. If the regulation of spread area, nuclear area and F-actin were due to a specific splice variant of nesprin-1 or nesprin-2 we would only expect to see a change with a siRNA that targets a specific exon; whereas here we observe the same alterations with siRNAs targeting different exons. Furthermore, nesprin-1 or nesprin-2 knockdown did not affect EC proliferation or cell adhesion (Supporting Information Fig. 3c and 3d), indicating that the responses are not an indirect consequence of reduced cell number or increased adhesion. Overall, our results indicate that nesprins play an important role in regulating EC morphology and actin cytoskeletal organization.

**Nesprin-1 and Nesprin-2 Regulate Nuclear Envelope Organization**

Nesprin-1 and nesprin-2 can link the actin cytoskeleton to the nuclear envelope [Mellad et al., 2011]. To determine whether nesprins affected nuclear envelope organization, we
stained ECs with antibodies to emerin, which is predominantly localized to the inner nuclear envelope and interacts with lamins as well as nesprin-1 and nesprin-2 [Haque et al., 2010; Berk et al., 2013]. Depletion of nesprin-1 or nesprin-2 induced a decrease in emerin localization to the nuclear envelope (Fig. 4 and Supporting Information Fig. 4b). Since all siRNAs to nesprin-1 and nesprin-2 lead to these changes (Fig. 4), this again is unlikely to be due to a specific nesprin-1 or nesprin-2 splice variant. Thus nesprins contribute to the morphology of both the nucleoskeleton and cytoskeleton as well as to the organization of components of these structures.

**Nesprin-1 and Nesprin-2 Regulate Endothelial Cell Migration**

We postulated that nesprins could play a role in the regulation of EC migration due to changes in the cytoskeleton.
induced by nesprin depletion. ECs do not grow or migrate as single cells in vitro, but preferentially form cell-cell adhesions. We therefore used a modified scratch wound assay (Oris assay), to test the effects of nesprins on cell migration (Fig. 5a). Traditional scratch wound assays suffer from several limitations: finding similar clear wound areas in each repeat and condition is technically challenging, scratching the dish can lead to removal of the plated matrix under the cells, and cell debris can affect the neighbouring cells. The Oris assay employed here resolves these issues as identical stoppers are placed into a well after matrix plating and then cells are seeded around the edge. This allows for a replicable clear area for each repeat and negates the effects of matrix removal and cell debris due to scratching. Using this assay nesprin-1 or nesprin-2 depletion significantly reduced EC migration with all siRNAs tested (Fig. 5b).

To investigate how nesprins might regulate EC migration, we analysed their morphology in more detail. The

**Fig. 3. Nesprin-1 and nesprin-2 regulate cell shape and nuclear morphology.** HUVECs were transfected with siRNAs targeting nesprin-1, nesprin-2 or a control siRNA as in Figure 1. After 72 h, cells were then fixed, stained with Alexa488-conjugated phalloidin, paxillin antibodies or DAPI. Scale bar: 100 μm. (a) Representative images of focal adhesion distribution shown by paxillin staining in transfected cells. Scale bar: 100 μm. (b) Cell area (μm²) was measured in ImageJ from F-actin-stained cells; n ≥ 56 cells/experiment in three independent experiments. Box and whisker plots, boxes show mean and 25th and 75th percentile, whiskers show 5th–95th percentile, outliers not shown; one-way ANOVA followed by Bonferroni multiple comparison’s test, *p < 0.05, **p < 0.01, ***p < 0.001. (c) Nuclear area was measured from DAPI staining in 35–50 cells in three independent experiments. Scatter plot displays mean ± SEM; one-way ANOVA followed by Bonferroni multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001.
Interestingly, we observed that nesprin-depleted cells had longer extensions, as measured by distance from the nucleus to the furthest extremities of each cell (Fig. 6a, 6b and Supporting Information Fig. 5a), without affecting the proportion of cells with extensions (Fig. 6c, Supporting Information Fig. 5b). The decreased migration together with longer extensions in nesprin-depleted cells could be due to reduced interaction between the cytoskeleton and nucleus, so that the nucleus can no longer be pulled forward to follow plasma membrane extensions.

**Nesprin-1 and Nesprin-2 Regulate Angiogenic Endothelial Loop Formation**

EC migration is important for angiogenesis. We used endothelial loop assays on Matrigel as an *in vitro* assay for...
angiogenesis [Goodwin, 2007] (Fig. 7a). Depletion of nesprin-1 or nesprin-2 reduced loop formation at 24 h after plating on Matrigel (Fig. 7b and Supporting Information Fig. 5c). In this assay, nesprin-1 depletion had a stronger effect than nesprin-2 depletion, although in all other experiments they had similar effects. Timelapse microscopy analysis indicated that ECs rarely divided during this assay (data not shown), making it unlikely that the difference is due to reduced proliferation.

**Discussion**

Through the roles of nesprins in the nuclear envelope LINC complex, nesprins link the cytoskeleton to the nucleus [Mellad et al., 2011]. Here we compare directly for the first time the functions of nesprin-1 and nesprin-2 in regulating morphology and migration in ECs. We show that depletion of either nesprin-1 or nesprin-2 increases EC spreading and stress fibre levels, yet inhibits EC migration and endothelial loop formation in an angiogenesis assay. We report that ECs express a number of different nesprin-1 and nesprin-2 isoforms, with the total mRNA levels of nesprin-1 and nesprin-2 being similar (Supporting Information Fig. 1). It is very unlikely that any single siRNA or exon-specific knockout in mice reduces the expression of all nesprin isoforms, and thus the results we report are probably due to depletion of a subset of isoforms that include the exons targeted by the siRNAs.

The increase in cell spread area and nuclear area upon nesprin knockdown most likely reflects a decrease in cellular tension due to detachment of actin filaments from the nucleus. The increase in stress fibres and hence F-actin levels observed in nesprin-depleted cells is probably a compensatory mechanism to increase cellular tension. Consistent with our results on nesprin-2 depletion in ECs, mice lacking the actin-binding CH1 domain of nesprin-2 have increased nuclear size in the epidermis, and altered emerin distribution [Luke et al., 2008]. Similarly, cardiomyocytes from mice lacking C-terminal spectrin repeat exons of nesprin-1 and nesprin-2 had increased nuclear area compared to wild-type controls [Banerjee et al., 2014]. Interestingly, we have found that the most abundant nesprin-2

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**Fig. 5. Nesprin-1 and nesprin-2 regulate endothelial cell migration.** HUVECs were transfected with siRNAs targeting nesprin-1, nesprin-2 or a control siRNA as in the previous figures. Cell migration was determined using Oris™ assays; each set of transfected cells were analysed in duplicate or triplicate in three independent experiments. (a) Representative images for Oris™ assays of transfected cells stained with Cell Tracker Orange (CMRA) dye, immediately after removing stoppers (0 h) and 24 h later. Scale bar: 1 mm. (b) Cell migration of transfected cells as determined by gap closure of Oris assay. Values are mean ± SEM; two-way Student’s t-test, **p < 0.01, ***p < 0.001, n = 3.
isoform in HUVECs is nesprin-2 giant, and thus it is likely that our siRNAs reduce the expression of this isoform.

However, a siRNA pool targeting nesprin-1 was reported to increase nuclear height in HUVECs, although in this case cell spread area and F-actin levels were not analysed [Chan

nelor et al., 2010]. It is possible the difference between these results and ours on nuclear shape reflects use of siRNAs targeting different regions of nesprin-1 and hence distinct nesprin-1 isoforms, and/or differences in HUVEC culture conditions.

Nesprin depletion has been shown to affect migration in other cellular systems, although how nesprins affect migration is not well understood [Chancellor et al., 2010; Mellad et al., 2011; Rashmi et al., 2012; Yu et al., 2011]. One possibility is that they act via the LINC complex to affect migration by regulating nuclear movement [Gundersen and Worman, 2013], and indeed the longer cellular extensions we observe in ECs depleted of nesprin-1 or nesprin-2 could reflect reduced migration of the nucleus in the direction of membrane protrusion. Mislocalization of the LINC complex component emerin could also contribute to the phenotype of nesprin-1/2-depleted ECs, since emerin can affect actin dynamics [Ho et al., 2013]. In addition, nesprins could normally limit cellular extensions by regulating the

**Fig. 6. Nesprin-1 and nesprin-2 alter extension length in endothelial cells.** HUVECs were transfected with siRNAs targeting nesprin-1, nesprin-2 or a control siRNA as in the previous figures. Images from cell area and F-actin intensity measurements were used for extension analysis. (a) Representative image for the analysis of extension length in transfected cells stained for phalloidin (same image as Fig. 1a). White lines on cells indicate the distance from nucleus to tip of protrusion. Scale bar: 100 μm. (b) Extension length and (c) extension number was measured in at least 41 cells/experiment in three independent experiments. Box and whisker plots, boxes show mean and 25th and 75th percentile, whiskers show 5th–95th percentile, outliers not shown; one-way ANOVA followed by Bonferroni multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001.
actin cytoskeletal tension across cells, thereby controlling cell migration. Alternatively, it has been suggested that nesprins regulate migration through focal adhesions. Nesprin-2 depletion in subconfluent HUVECs and keratinocytes from nesprin-2 giant isoform knockout mice have increased numbers of integrin-based focal adhesions [Chan-celler et al., 2010; Rashmi et al., 2012]. Notably, some short nesprin-1 and nesprin-2 isoforms localize to focal adhesions [Rajgor et al., 2012]. However, nesprin-1/2 depletion did not detectably affect the localization of the focal adhesion protein paxillin in ECs. This suggests that only a subset of nesprin-1 and nesprin-2 isoforms affect focal adhesions, which are not targeted by siRNAs used in this study.

Our results indicate that both nesprin-1 and nesprin-2 play a role in angiogenic loop formation, with nesprin-1 having the strongest effect. In the loop angiogenesis assay, loops still form, however there are fewer loops following nesprin-1 or nesprin-2 depletion as each loop formed is longer. Effects of nesprins on angiogenesis in vivo have not yet been investigated; however these data suggest that nesprins could regulate this process. Angiogenesis contributes to atherosclerosis and cancer progression, and thus it would be interesting to study angiogenesis models in mice containing genetically modified nesprin-1 or nesprin-2 loci [Luke et al., 2008; Puckelwartz et al., 2009].

It is interesting that knockdown of either nesprin-1 or nesprin-2 leads to a similar cellular phenotype in all the assays we tested, which implies that they have different non-redundant functions in cells and thus are each unable to compensate for loss of the other nesprin. Nesprin-1 has previously been shown to affect cell migration in ECs [Chancellor et al., 2010], consistent with our results; however the role of nesprin-2 in ECs has not previously been investigated.

However, there were differences in the extent of response to each individual nesprin knockdown. For example, nesprin-1 depletion had a bigger effect than nesprin-2 depletion on angiogenic loop numbers. Thus some responses are more dependent on the function of one of the two nesprins. It will be interesting to determine whether this reflects distinct roles of the different endothelial isoforms of nesprin-1 and nesprin-2 [Rajgor et al., 2012; Randles et al., 2010]. Interestingly nesprin-3 depletion induces elongation of human aortic ECs and is required for flow-induced migration and polarization of the centrosomes [Morgan et al., 2011]. In contrast to nesprin-1 and nesprin-2, nesprin-3 links intermediate filaments to the nucleus, indicating that multiple cytoskeletal elements need to interact with the nucleus to maintain cell migration.

Our results indicate that both nesprin-1 and nesprin-2 are critical for regulation of EC migration, probably by linking the actin cytoskeleton and nucleus, and thereby controlling cellular tension and cellular and nuclear spreading. Additionally, nesprins affect angiogenic loop formation, which could reflect their roles in cellular tension and

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**Fig. 7. Nesprin-1 and nesprin-2 regulate angiogenic loop formation.** HUVECs were transfected with siRNAs targeting nesprin-1, nesprin-2 or a control siRNA. Cells were seeded onto a thick Matrigel layer, and fixed after 24 h. Cells were stained with Alexa546-conjugated phalloidin (a) Representative images for the analysis of loop length. Scale bar: 1 mm. (b) The number of loops was quantified. For each set of transfected cells, six images were acquired and analysed from each of three independent experiments. Box and whisker plots, boxes shows mean and 25th and 75th percentile, whiskers show 5th–95th percentile, outliers not shown; one-way ANOVA followed by Bonferroni multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001.
migration. Our results using multiple siRNAs also suggest that this tethering function of both nesprin-1 and nesprin-2 may not be solely dependent on the giant isoforms. It is likely that other shorter nesprin isoforms can interact with actin filaments via some of their spectrin repeat domains, as has been described for other spectrin repeat-containing proteins [Zhang et al., 2001]. The functions of nesprins in ECs could be important for EC responses in cardiovascular diseases and cancer, including EC migration following ischaemic damage and angiogenesis in atherosclerotic plaques, and angiogenesis in cancer.

Materials and Methods

Cell Culture and Transfection

Human umbilical vein endothelial cells (HUVECs) were from Lonza or Promocell. Cells were cultured at 37°C and 5% CO₂ in EBM-2 containing 2% FCS and EGM-2 growth supplements (Lonza). Cells were cultivated on dishes coated with 10 μg/ml human fibronectin (Calbiochem). Cells were used for assays between passages 2 and 4. All siRNA transfections were carried out using Oligofectamine (Invitrogen) according to the manufacturer's instructions. siRNAs (Supporting Information Table 1) were obtained from Dharmacon or Eurogentec. Cells were plated at 10⁵ cells in each well of 6-well plates and incubated overnight. Cells were then transfected with 5 nmol siRNA in Optimem (Invitrogen). Cells were analysed 72 h after transfection.

Quantitative PCR

Total RNA was isolated from cells using Trizol and phenol/ chloroform extraction or with an RNeasy kit (Qiagen). Total RNA (1–2 μg) was reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. siRNAs (Supporting Information Table 1) were obtained from Dharmacon or Eurogentec. Cells were plated at 10⁵ cells in each well of 6-well plates and incubated overnight. Cells were then transfected with 5 nmol siRNA in Optimem (Invitrogen). Cells were analysed 72 h after transfection.

Immunofluorescence, Confocal Microscopy and Cell Measurements

HUVECs were plated onto fibronectin-coated coverslips 48 h after transfection, and incubated at 37°C for 24 h. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% or 0.2% Triton X-100 in PBS. Cells were blocked in Tris-buffered saline (TBS) containing 3% BSA and then incubated with Alexa488- or Alexa546-conjugated phalloidin (Invitrogen, 1:400), anti-paxillin antibody (mouse, 1:200, BD Biosciences), DAPI (Invitrogen, 1:10,000) and/or anti-emerin antibody (mouse, 1:40, Vector Laboratories Ltd) followed by Alexa488- or Alexa568-conjugated anti-mouse IgG (Invitrogen, 1:1000) in 1% BSA/TBS as indicated. After staining, cells were washed in PBS and distilled water and then mounted onto slides using Fluorsave™ (Calbiochem) or Dako Fluorescent (Invitrogen).

Images for cell area, F-actin intensity and extension length were acquired using an A1R confocal microscope (Nikon) with a CFI Plan Fluor 40x oil objective. Images for nuclear area and focal adhesions (paxillin staining) were acquired using a LSM510 confocal microscope (Zeiss) with an EC Plan-Neofluar 40×1.30 oil objective.

For cell area measurements, 10 images of phalloidin-stained cells per sample were analysed per experiment. At least 56 cells were analysed per experiment in three independent experiments. Cells were manually traced and area was measured using ImageJ. For nuclear area measurements, 35–50 cells were analysed. DAPI staining was used to manually trace the nuclear area using ImageJ. Four representative cells stained for emerin and DAPI in each image were chosen and fluorescence profiles were generated using ZEN 2011 software (Zeiss). To quantify emerin distribution, cells were classified as ‘None’ (staining only in the nucleolus), ‘Dim’ (nuclear membrane stained stronger than rest of nucleus but with still clear staining in the middle) or ‘Bright’ (nuclear membrane and rest of nucleus with similar intensity). The analysis was carried out blinded by a person independent of the experiment.

For quantification of F-actin intensity, phalloidin staining was imaged using identical laser settings between samples to permit comparison. Look-up tables were used to ensure that pixel saturation was not reached and mean grey intensity per pixel was quantified using ImageJ following common background subtraction. Stress fibres were quantified using the phalloidin-stained images for actin intensity and manually assigning a score to each cell. Quantification was done by assigning a score to each cell based on the stress fibre content in the centre of the cell. At least 50 cells were analysed in at least two independent experiments. For
Western Blotting

Cells were lysed by scraping into sample buffer (NuPAGE 4× SDS sample buffer; Invitrogen). The proteins were separated on precast NuPAGE 4–12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose membrane (Immobilon), and incubated with antibodies in PBS containing 3% non-fat milk powder. Antibodies used were mouse anti-paxillin (1:5000, BD Biosciences), mouse anti-vinculin (1:500, Sigma), and HRP-conjugated sheep anti-mouse antibodies (1:1000; GE Healthcare).

Proliferation Assay and Cell Adhesion Assays

For the proliferation assay, for each siRNA 4.8 × 10⁴ transfected HUVECs were plated and incubated at 37°C for 24 h. After incubation the cells were trypsinized and counted using a hemocytometer. The percentage change in cell number between 0 and 24 h was calculated.

For cell adhesion; wells of a 96-well plate were coated with fibronectin for 1 h then blocked in 0.5% BSA in medium for 1 h. HUVECs (4 × 10⁴/well) were plated in quadruplicate for each condition. The cells were incubated for 30 min at 37°C, shaken for 15 s at 2000 rpm and washed with 0.1% BSA in medium. The cells were then fixed in 4% paraformaldehyde in PBS, stained with crystal violet (5 mg/ml in 2% ethanol) for 10 min, then washed with water and left to dry. After incubation with 2% SDS, crystal violet staining was quantified at 540 nm using a GENios Pro plate reader. All measurements were analysed and statistics performed using Microsoft Excel and GraphPad Prism.

Migration Assay

Twenty-four hours after transfection of HUVECs with siRNAs, cells were labelled with 2 μM Cell Tracker Orange (CMRA) dye (Invitrogen Molecular Probes) for 40 min at 37°C. The dye-containing medium was removed, replaced with fresh medium and cells incubated for a further 40 min at 37°C. Wells of a 96-well plate were coated with fibronectin (10 μg/ml) and Oris™ cell seeding stoppers placed in the centre of each well. HUVECs were seeded at 4 × 10⁴ cells per well and incubated at 37°C. Each condition was present in triplicate wells. After 24 h, the stoppers were removed, wells washed with medium, and an image was acquired (t = 0 h). The cells were incubated for a further 24 h and then a second image (t = 24 h) was acquired of the same position as the first image. Images were generated by multi-field phase contrast imaging using an Olympus IX81 inverted microscope (Olympus Inc., PA, USA) with a 4× UPLFLN 4XPH objective (Olympus Inc., PA, USA), or using a Nikon TE2000-E microscope with a Plan Fluar 4x objective (Nikon) and a Hamamatsu Orca-ER digital camera. Images were analysed using ImageJ (National Institutes of Health, USA). The zone where no cells were attached due to the stoppers was marked in the t = 0 h image, and the filter was applied to the t = 24 h image. The migration was calculated as the difference in % area of fluorescence between the t = 0 h and t = 24 h image, after setting a threshold using the t = 0 h image.

Endothelial Angiogenic Loop Formation Assay

Matrigel (10 mg/ml, without phenol red, BD Biosciences) was diluted 1:1 in PBS (containing calcium and magnesium) and 300 μl added to each well of a 6-well plate and incubated at room temperature for 90 min. Forty-eight hours after transfection, 2 × 10⁵ cells were plated per well onto the Matrigel and incubated at 37°C for 24 h. Cells were then fixed and stained with Alexa546-conjugated phalloidin. Images were acquired using phase contrast multi-field imaging using an Olympus IX81 inverted microscope (Olympus Inc., PA, USA) with a 4× UPLFLN 4xPH objective (Olympus Inc., PA, USA). In each experiment, six images were acquired per well. Loops (an unbroken loop of cells) were counted using ImageJ and analysed using Microsoft Excel and GraphPad Prism.

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