Rnd3 induces stress fibres in endothelial cells through RhoB

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Summary

Rnd proteins are atypical Rho family proteins that do not hydrolyse GTP and are instead regulated by expression levels and post-translational modifications. Rnd1 and Rnd3/RhoE induce loss of actin stress fibres and cell rounding in multiple cell types, whereas responses to Rnd2 are more variable. Here we report the responses of endothelial cells to Rnd proteins. Rnd3 induces a very transient decrease in stress fibres but subsequently stimulates a strong increase in stress fibres, in contrast to the reduction observed in other cell types. Rnd2 also increases stress fibres whereas Rnd1 induces a loss of stress fibres and weakening of cell–cell junctions. Rnd3 does not act through any of its known signalling partners and does not need to associate with membranes to increase stress fibres. Instead, it acts by increasing RhoB expression, which is then required for Rnd3-induced stress fibre assembly. Rnd2 also increases RhoB levels. These data indicate that the cytoskeletal response to Rnd3 expression is dependent on cell type and context, and identify regulation of RhoB as a new mechanism for Rnd proteins to affect the actin cytoskeleton.

Key words: Rho GTPases, Endothelial cells, Actin filaments, Rnd proteins

Introduction

Endothelial cells (EC) line blood vessels and play an essential physiological role in forming a barrier between the blood and the tissues and mediating the transfer of nutrients. During inflammation, EC mediate the passage of solutes and leukocytes from the blood into the tissues (Nourshargh et al., 2010). In response to pro-inflammatory cytokines, EC reorganize their actin cytoskeleton to form stress fibres, which exert tension on cell–cell junctions leading to increased vascular permeability (Bogatcheva and Verin, 2008; Millán et al., 2010). This response is mediated by Rho GTPases, which are well known to regulate cytoskeletal dynamics (Ridley, 2011; Spindler et al., 2010). In particular, the closely related isoforms RhoA, RhoB and RhoC can each induce stress fibres when overexpressed, and all three isoforms contribute to the formation of stress fibres (Aktories and Just, 2005; Melendez et al., 2011).

The three Rnd proteins, Rnd1, Rnd2 and Rnd3/RhoE, are a subfamily of the Rho family with unusual properties and functions (Riou et al., 2010). Most Rho GTPases cycle between an active GTP-bound and inactive GDP-bound conformation, and are downregulated by GTP hydrolysis (Heasman and Ridley, 2008). However, Rnd proteins are unable to hydrolyse GTP due to amino acid substitutions that prevent this enzymatic activity. For Rnd3, there is good evidence that it is regulated at the transcriptional level by a wide variety of stimuli (Riou et al., 2010). In addition, it has been shown to be phosphorylated on multiple sites by the serine/threonine kinases ROCK1 and PKCα, which leads to its stabilisation and translocation from membranes to the cytosol (Madigan et al., 2009; Riento et al., 2005). Rnd1 and Rnd3 induce loss of stress fibres and antagonise Rho/ROCK signalling in a variety of cell types (Riou et al., 2010). They act in part by stimulating the activity of p190RhoGAP, a GTPase-activating protein that downregulates RhoA activity in cells (Wennerberg et al., 2003). Rnd2 on the other hand can stimulate cell contraction through activation of RhoA (Tanaka et al., 2006). Rnd proteins have been implicated in cell migration, for example Rnd2 and Rnd3 contribute to cortical neuron migration *in vivo* (Heng et al., 2008; Pacary et al., 2011). Rnd proteins can interact with a variety of downstream targets to induce cellular responses (Riou et al., 2010).

Here we investigate the functions of the three Rnd proteins in EC. Surprisingly, we find that Rnd3 induces an increase in stress fibres, in contrast to its ability to induce loss of stress fibres in other cell types. Rnd2 also increases stress fibres and stimulates cell contraction and membrane blebbing. Rnd2 and Rnd3 increase RhoB expression and Rnd3 requires RhoB for stress fibre induction. By contrast, Rnd1 induces stress fibre disassembly. Each Rnd protein also affects endothelial cell–cell junctions. Our results show for the first time that each of the three Rnd proteins induces a distinct phenotype in a single cell type, and that the response to Rnd3 is different in EC to other cell types.

Results

Rnd3 induces stress fibres in endothelial cells

To compare the functions of Rnd proteins in EC, we investigated the effects of expressing Rnd1, Rnd2 and Rnd3 on the actin cytoskeleton and cell–cell junctions in human umbilical cord

endothelial cells (HUVECs). HUVECs express endogenous Rnd1, Rnd2 and Rnd3 mRNAs (Fig. 1A). Control confluent HUVECs had strong cortical F-actin around the periphery at cell–cell junctions, and some stress fibres traversing the cytoplasm (Fig. 1B). The adherens junction protein VE-cadherin was localised predominantly linearly along cell–cell adhesions or in a reticular network, as previously described (Fernández-Martin et al., 2012; Millán et al., 2010). The integrin-associated focal adhesion protein paxillin localised to small focal contacts, which were concentrated in regions where there were strong F-actin bundles (Fig. 1C). Rnd1 and Rnd3 have previously been shown to induce loss of stress fibres and reduce contractility in a variety of cell types (Riou et al., 2010). Rnd1 induced a decrease in stress fibres in some HUVECs, and a reduction in focal contacts at 24 hours after transfection (Fig. 1C). Interestingly, some Rnd1-expressing cells extended protrusions under neighbouring cells (Fig. 1C, arrow). Surprisingly, we found that both Rnd2 and Rnd3 induced an increase in stress fibres and paxillin-containing focal adhesions in endothelial cells (Fig. 1B,C). This correlated with disruption of linear VE-cadherin localisation along cell–cell junctions, particularly in regions at the ends of stress fibres. Stress fibres were attached to cell–cell junctions (Fig. 1B, magnified images), as previously described in TNFα-stimulated HUVECs (Millán et al., 2010).

To determine the time course of the responses to Rnd proteins, we compared cells 10 hours and 24 hours after transfection (Fig. 2). Rnd1 induced a decrease in stress fibres at both time points. Adherens junctions were disrupted between Rnd1-expressing cells at both 10 and 24 hours (asterisk, Fig. 2A). Rnd2-expressing cells showed an increase in stress fibres at 10 hours, which was considerably stronger at 24 hours. At 24 hours, some Rnd2-expressing cells overlapped, or were extruded above, neighbouring cells (arrowhead, Fig. 2B). At 10 hours they still had adherens junctions with neighbours, but these were much reduced at 24 hours. Rnd3-expressing cells had an increase in stress fibres at 10 hours and 24 hours, and showed zipper-like discontinuous adherens junctions in areas where stress fibres were perpendicular to cell–cell junctions (arrows, Fig. 2A,B). The induction of stress fibres by Rnd3 was specific to EC, since both Rnd3 and Rnd1 induced loss of stress fibres in HeLa cells (supplementary material Fig. S1).

Cytoskeletal responses to Rnd protein expression in subconfluent endothelial cells

To determine whether the responses to Rnd proteins were modulated by cell–cell junctions, we tested their effects in subconfluent HUVECs. Changes in stress fibre levels were compared with surrounding untransfected cells. As a control for transfection, expression of GFP did not discernably affect the levels of stress fibres (Fig. 3A). Rnd1 induced a much stronger response in subconfluent cells than in confluent cells: stress fibres decreased, the cell body rounded up and cells had multiple long narrow branched protrusions (Fig. 3B). This response was already maximal 4 hours after transfection, and persisted to 10 hours. By 24 hours after transfection some of the cells had re-spread although they still had a strong reduction in stress fibres (Fig. 3B).

In subconfluent EC, Rnd2 rapidly induced stress fibres and cell contraction (Fig. 3C). Rnd3 also induced stress fibres at later time points (10 and 24 hours), but at 4 hours Rnd3 induced a slight decrease in stress fibres (Fig. 3B). This indicates that Rnd3 induces a transient response of loss of stress fibres and subsequently stimulates stress fibres at later time points.

Rnd3 induces stress fibres through Rho/ROCK signalling

Since the induction of stress fibres in EC by Rnd3 was unexpected, we explored further the molecular pathway underlying this response. ROCK kinase inhibitors prevent stress

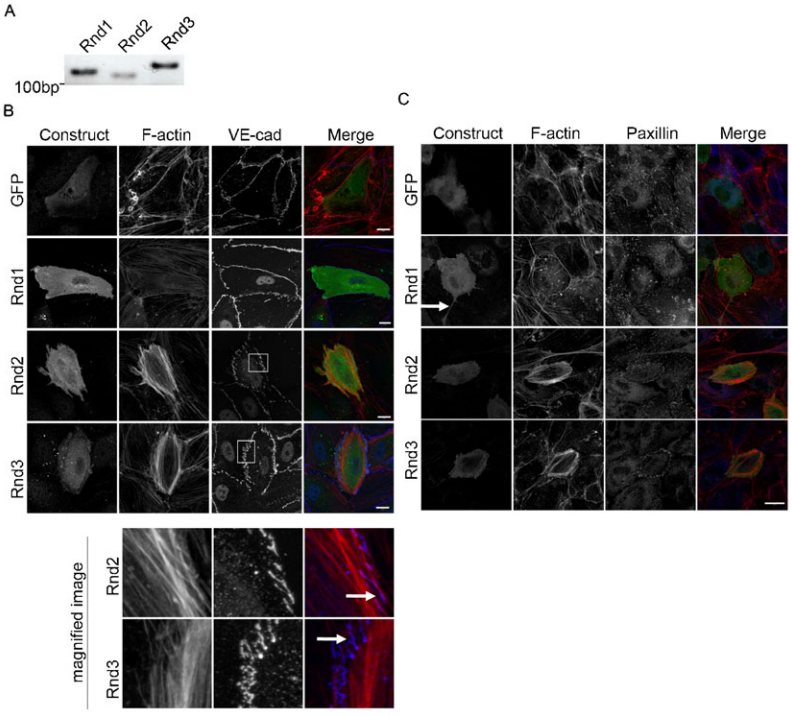

Fig. 1. Rnd2 and Rnd3 induce stress fibres whereas Rnd1 reduces stress fibres in endothelial cells. (**A**) Rnd mRNAs are expressed in HUVECs. Total RNA was isolated and reverse transcribed into cDNA. Rnd1, Rnd2 and Rnd3 were detected by PCR. Amplification products were separated on an agarose gel. (**B**,**C**) HUVECs were transfected with Rnd1, Rnd2, Rnd3 or GFP-encoding plasmids and fixed after 24 hours. Cells were stained for F-actin and with anti-Flag antibody to detect Rnd proteins and anti-VE-cadherin (B) or anti-paxillin (C) antibodies. (B) Images show maximum intensity projections of 11 confocal Z-stacks. Boxed regions (merge images) are shown magnified below. Scale bars: 10 µm. (C) Single confocal images. Scale bar: 20 µm.

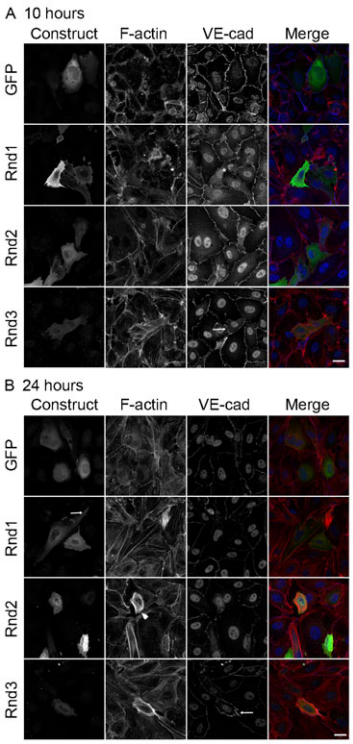

Fig. 2. Endothelial responses to Rnd protein expression are time dependent. HUVECs were transfected with Rnd1, Rnd2, Rnd3 or GFP-encoding plasmids and seeded at confluence. (**A**,**B**) Cells were fixed after 10 hours (A) or 24 hours (B), and then stained with anti-Flag antibody and for F-actin. Figures show representative images for both time points. Images show maximum intensity projections of 11–13 confocal Z-stacks. Scale bars: 20 µm.

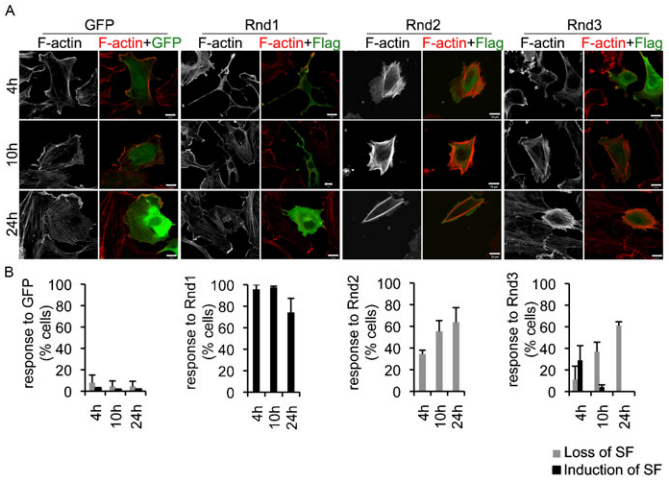

Fig. 3. Rnd proteins induce stronger responses in subconfluent endothelial cells. HUVECs were transfected with Rnd1, Rnd2, Rnd3 or GFP-encoding plasmids. (**A**) Cells were fixed after 4, 10 or 24 hours. Cells were stained for F-actin and with anti-Flag antibody to identify transfected cells. Scale bars: 10 µm. (**B**) Transfected cells were scored for an increase or decrease in stress fibres and membrane blebbing compared to surrounding untransfected cells. The bars represent the mean ± s.d. of 3 independent experiments; at least 68 cells were scored per time point and per experiment.

fibre formation in EC in response to a variety of stimuli (Beckers et al., 2010). The ROCK inhibitor H1152 inhibited Rnd3-induced stress fibres (Fig. 4A,B). We next tested whether Rnd3 required any of the Rho isoforms to induce stress fibres. RhoA is known to stimulate stress fibre formation in EC (Wójciak-Stothard et al., 1998) and the closely related RhoB and RhoC proteins can also induce stress fibres in other cell types (Aktories and Just, 2005). We found that RhoA, RhoB and RhoC could all induce stress fibres in EC (supplementary material Fig. S2). Treatment of HUVECs with C3 transferase, which ADP-ribosylates and inhibits all three Rho isoforms (Vogelsgesang et al., 2007), inhibited Rnd3-induced stress fibre assembly (Fig. 4C).

Mutational analysis shows that only the Rnd3 core GTP-binding domain is required for stress fibre induction

We next carried out mutational analysis to identify which regions of Rnd3 were required for it to induce stress fibres. Rnd proteins have N-terminal and C-terminal extensions compared to Rho, Rac and Cdc42 isoforms (Aspenström et al., 2007), outside of the core GTP-binding domain (Fig. 4D). Only the core GTP-binding domain of Rnd3 is required for stress fibre induction, since deletion of the Rnd3 N- and C-terminal regions or replacing the C-terminal region of Rnd3 with the equivalent Rnd1 or Rnd2 region did not affect the response significantly. Importantly, this indicates that C-terminal farnesylation of Rnd3 is not required for the induction of stress fibres, whereas it is required for Rnd3-induced loss of stress fibres (Roberts et al., 2008). A Rnd1/Rnd3 chimera with the Rnd3 C-terminal region did not induce stress fibres, underscoring the importance of the Rnd3 core GTP-binding domain for the response (Fig. 4D).

We previously identified 7 Ser/Thr phosphorylation sites on Rnd3, which can be phosphorylated by ROCK1 and/or PKC (Madigan et al., 2009; Riento et al., 2005). Phosphorylation was not required for induction of stress fibres, since mutation of these 7 residues from Ser/Thr to Ala (Rnd3 AllA) did not alter the response. Similarly, Rnd3 mutants that are unable to interact with ROCK1 (Rnd3 T173R, Rnd3 V192R) (Komander et al., 2008) still induced stress fibres (Fig. 4D). Residues Thr55 and/or Tyr60 in the Switch 1 region (also known as the effector domain) (Garavini et al., 2002), are known to be required for interaction with p190RhoGAP and other Rnd3 effectors (Riou et al., 2010; Wennerberg et al., 2003). However, they were not required for Rnd3 to induce stress fibres. In contrast, mutation of Thr37 (Rnd3 T37N) prevented stress fibre induction. This residue is critical for GTP binding and Rnd3 T37N does not induce cell cycle arrest or loss of stress fibres, indicating that it is completely inactive (Garavini et al., 2002; Villalonga et al., 2004). Taken together, these results show that only the core GTP-binding domain of Rnd3 is required for stress fibre induction, and that this is not mediated by known Rnd3 effectors. This is perhaps not surprising since these effectors have been characterised to mediate loss of stress fibres.

Rnd3 cytoplasmic localization is required for stress fibre induction

Although deletion of the Rnd3 C-terminus did not affect stress fibre assembly, mutations in the C-terminal CAAX box (the site for farnesylation and hence membrane association) reduced Rnd3-induced stress fibre induction (Fig. 5). These mutants were Rnd3-C241S, which lacks the cysteine to which the farnesyl group is added, and Rnd3-ΔCAAX, which lacks the last 4 amino acids of Rnd3. Both of these mutants localised preferentially to

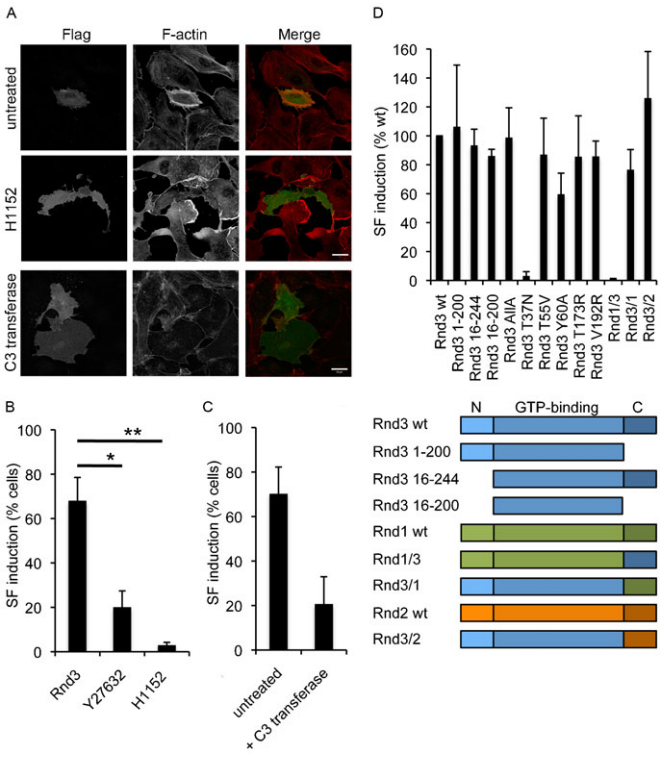

Fig. 4. Rho and ROCK inhibition prevent Rnd3-induced stress fibre formation. (**A**) HUVECs were transfected with plasmid encoding Flag-tagged Rnd3. After 16 hours, cells were treated with the ROCK inhibitors H1152 (20 μM) or Y27632 (10 μM) for an additional 6 hours. C3 transferase (1 μg/ml) was added to the cells 6–8 hours after transfection for an additional 16 hours. The cells were then fixed and stained for F-actin and with anti-Flag antibody to detect Rnd3-expressing cells. Scale bars: 20 μm. (**B**,**C**) HUVECs treated and stained as in A were scored for an increase in stress fibre formation compared to untransfected cells. Data in B represent the mean ± s.d. of 3 independent experiments; at least 100 cells were counted per experiment. *$P \leq 0.05$, **$P \leq 0.01$, Student's *t*-test. Data in C represent the mean ± s.d. of 2 independent experiments; at least 80 cells were counted per experiment. (**D**) HUVECs were transfected with constructs encoding wild-type Flag-Rnd3 (Rnd3 wt) or the indicated Rnd3 mutants and Rnd3/Rnd2 or Rnd3/Rnd1 chimerae. Schematic representation of the mutants and chimerae is shown below. After 24 hours, cells were fixed and stained for F-actin and with anti-Flag antibody. Transfected cells were scored for increased stress fibre levels compared to surrounding untransfected cells, and are shown as a % of Rnd3 wt-expressing cells. Data represent the mean ± s.d. of 2 independent experiments; at least 24 cells were counted per experiment.

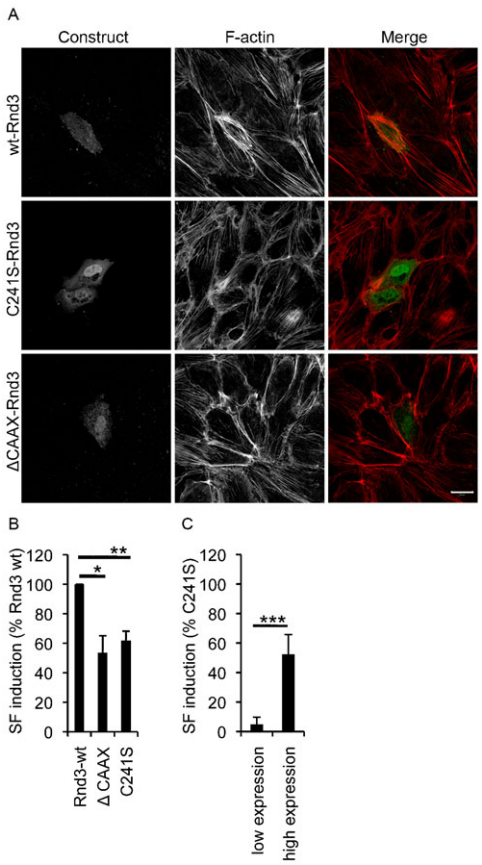

Fig. 5. Mutation of the CAAX-box reduces Rnd3-induced stress fibre formation. (**A**) HUVECs were transfected with wild-type Rnd3 or the indicated Rnd3 CAAX box mutants. After 24 hours the cells were fixed and stained for F-actin and with anti-Flag antibody to detect transfected cells. Scale bar: 20 μm. (**B**,**C**) Quantification of cells showing an induction of stress fibre formation. The cells were scored for an increase in stress fibres compared to untransfected cells. B shows % of Rnd3-expressing cells with stress fibres compared to Rnd3 wt. Data represent the mean ± s.d. of 3 independent experiments; at least 40 cells were counted per experiment. C quantifies % of Rnd3-C241S-expressing cells with high or low levels of Rnd3 in the cytoplasm that have stress fibres. Data represent the mean ± s.d. of 5 independent experiments; at least 35 cells were scored per experiment. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

the nucleus (Fig. 5A), because of a C-terminal cryptic nuclear localisation signal that is normally masked by farnesylation (Foster et al., 1996). We therefore investigated whether Rnd3 nuclear localisation correlated with stress fibre induction. Interestingly, cells with the highest level of Rnd3-C241S in the cytoplasm had the highest level of stress fibre induction (Fig. 5B). This implies that Rnd3 needs to be in the the cytoplasm to induce stress fibres.

Rnd3 acts through RhoB to induce stress fibres and induces RhoB expression

Since C3 transferase inhibited the Rnd3 response, we investigated which of the three Rho isoforms was important for the Rnd3 response by depleting RhoA, RhoB or RhoC with siRNAs. Depletion of RhoB most strongly reduced the induction of stress fibres by Rnd3, although RhoA depletion also significantly reduced the Rnd3 response (Fig. 6A–C). As previously described for other cell types (Ho et al., 2008; Vega et al., 2011), RhoA depletion increased the expression of RhoB in EC (Fig. 6B,C). Knockdown of RhoB and RhoA together further reduced Rnd3-induced stress fibres compared to RhoB alone, at least for RhoB siRNA#2, which was less efficient at depleting RhoB than RhoBsiRNA#1. This indicates that the effect of RhoA depletion is not due to RhoB upregulation. Unfortunately knockdown of RhoC together with exogenous Rnd3 expression was toxic to EC, and thus it was not possible to address the role of RhoC in Rnd3 responses, although RhoC depletion alone did not affect cell viability and did not decrease stress fibres (data not shown).

Since Rnd3 induction of stress fibres required RhoB, and the Rnd3 response is delayed (initially inducing a decrease in stress fibres at early time points) (Fig. 3B), we investigated whether any of the Rnd proteins altered RhoB expression. RhoB is known to be rapidly induced at the transcriptional and protein level by a variety of stimuli, including growth factors and DNA damage (Wheeler and Ridley, 2004). Transfection of HUVECs with Rnd3

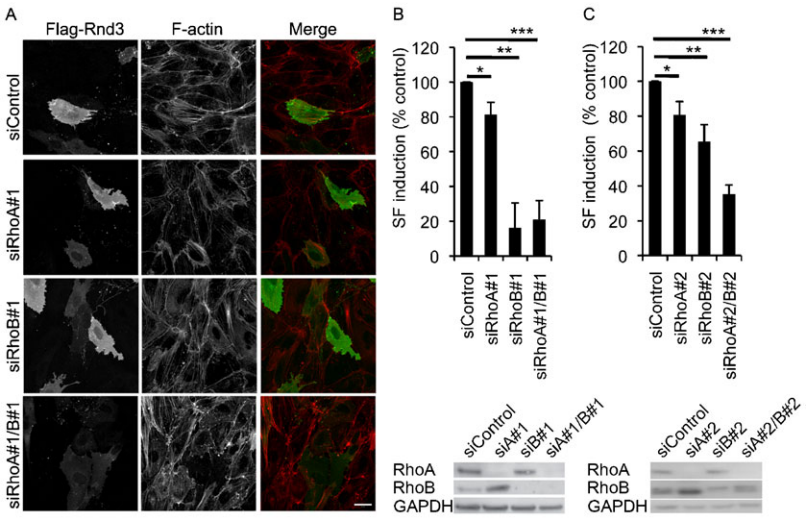

Fig. 6. RhoB is required for Rnd3-induced stress fibre formation. (**A**) HUVECs were transfected with siRNAs against RhoA or RhoB either individually or in combination. After 48 hours, cells were transfected with plasmid encoding Flag-Rnd3. After 24 hours, cells were fixed and stained with anti-Flag antibody and for F-actin. (A) Representative images of cells. Scale bar: 20 μm. (**B**,**C**) Cells were scored for an increase in stress fibre formation compared to untransfected cells. Results for 2 different siRNAs targeting RhoA or RhoB are shown. Western blots showing levels of RhoA and RhoB proteins 72 hours after siRNA transfection are shown below. Data represent the mean ± s.d. of 3 independent experiments; at least 24 cells were counted per experiment. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

and Rnd2 but not Rnd1 induced RhoB protein expression (Fig. 7).

Discussion

Rnd proteins are well known to induce changes to the actin cytoskeleton, but responses to each of the three isoforms have not previously been compared directly in one cell type. Here we show that each Rnd protein induces a distinct response in EC. Rnd1 induces loss of stress fibres and cell–cell junctions. Rnd2 stimulates actomyosin contractility and cell rounding and membrane blebbing, and this leads to a decrease in cell–cell interaction. Rnd3 on the other hand transiently decreases stress fibre levels and then increases stress fibres, and only occasionally induced membrane blebbing. Both Rnd2 and Rnd3 increase RhoB levels, and RhoB is important for Rnd3-induced stress fibre formation.

Of the Rnd proteins, Rnd2 is the most potent inducer of contractility and stimulates membrane blebbing. It is likely that Rnd2 and Rnd3 act through a similar mechanism in EC to induce actomyosin contractility, but Rnd2 induces the strongest response, perhaps because it cannot effectively interact with p190RhoGAP and downregulate RhoA activity, whereas Rnd1 and Rnd3 can both act through this pathway (Wennerberg et al., 2003). Presumably Rnd3 transiently acts through p190RhoGAP in EC when it initially induces a decrease in stress fibres, but subsequently this effect is masked by the increased stress fibre formation. It is also likely that the Rnd1/Rnd3-induced decrease in RhoA activity is downregulated through a negative feedback loop. Indeed, the endothelial rounding response to Rnd1 is reduced at later time points, when cells re-spread. Similarly, induction of Rnd3 expression only transiently induces a loss of stress fibres and rounding in fibroblasts; by 24 hours after induction, cells had flattened out again (Villalonga et al., 2004).

We observe that Rnd3 is able to induce stress fibres independent of membrane localisation. This is a surprising result, since it is generally assumed that prenylation and hence membrane association is required for Rho protein signalling. Indeed, Rnd3 prenylation is required for it to induce loss of stress fibres (Roberts et al., 2008). Interestingly, Rnd2 appears to localise predominantly in the cytosol and not on the plasma membrane, although it might partly be associated with intracellular membrane compartments (Roberts et al., 2008). This localisation may underlie its strong ability to induce stress fibres and contractility in EC.

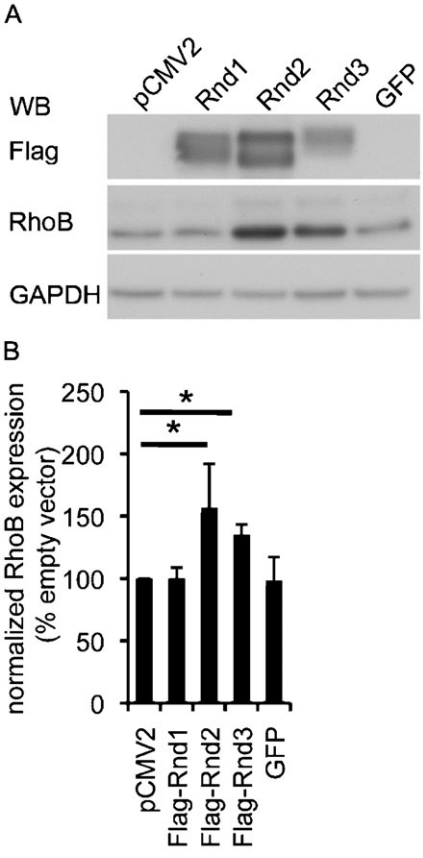

Fig. 7. Expression of Rnd2 and Rnd3 induce RhoB upregulation. HUVECs were transfected with 10 μg of the indicated plasmids. After 10 hours the cells were lysed, proteins separated by SDS-PAGE and immunoblotted with antibodies to Flag to detect Rnd protein expression, RhoB antibodies, and GADPH antibodies as a loading control. A representative blot (**A**) and the quantification for 3 independent blots (**B**) are shown. RhoB protein expression was normalised to GAPDH and is shown as % of control vector (pCMV2)-transfected cells (B). Data represent the mean ± s.d.; *$P \leq 0.05$.

Rnd2 and Rnd3 act through RhoB to induce stress fibres. RhoA, RhoB and RhoC can all induce stress fibres when overexpressed, and it has been reported that all three isoforms need to be depleted to induce loss of stress fibres in mouse embryonic fibroblasts (Melendez et al., 2011). Here we find that RhoB is the predominant isoform acting downstream of Rnd2 and Rnd3 to induce stress fibres, and indeed it is upregulated at the protein level. The RhoB gene is highly regulated at both the transcriptional and translational level (Huang and Prendergast, 2006). Interestingly, in EC the microRNA miR-21 decreases RhoB expression, and this correlates with reduced stress fibre levels and inhibition of endothelial migration (Sabatel et al., 2011). This indicates that RhoB has a significant role in regulating stress fibre levels in endothelial cells. Whether Rnd2 and Rnd3 induce mRNA or protein stabilisation remains to be established.

In summary, we report a novel function of Rnd3 in stimulating stress fibre formation in EC, in contrast to its previously described role in reducing stress fibres in other cell types. In addition, we have demonstrated a crucial role for RhoB in mediating the response to Rnd3.

Materials and Methods

Antibodies and reagents

Antibodies were obtained from the following sources: mouse anti-GADPH (Millipore), mouse anti-VE-cadherin and mouse anti-paxillin (BD Bioscience), mouse and rabbit anti-Flag epitope (Sigma–Aldrich), mouse anti-RhoA, rabbit anti-RhoB and goat anti-andRhoC (Santa Cruz). Mouse anti-Rnd3/RhoE was previously described (Riento et al., 2003). Secondary HRP-conjugated antibodies were from DakoCytomation. Alexa Fluor 488, 546 and 647-labelled secondary antibodies and Alexa Fluor 546-labelled phalloidin were from Molecular Probes. siRNAs were obtained from Dharmacon (ThermoScientific); sequences are in supplementary material Table S1.

Plasmids and mutagenesis

The following cDNAs, all in pCMV5-Flag, have been previously described: Rnd3 (Riento et al., 2003), Rnd3-AllA (Riento et al., 2005), $Rnd3^{T37N}$ (Villalonga et al., 2004), $Rnd3^{T173R}$, $Rnd3^{V192R}$ (Komander et al., 2008), and Rnd2 (Pacary et al., 2011). Rnd1, $Rnd3^{1–200}$, $Rnd3^{16–244}$, $Rnd3^{1–200}$, $Rnd3^{\Delta CAAX}$, Rnd1/3 ($Rnd1^{1–190}$/$Rnd3^{201–244}$) and Rnd3/1 ($Rnd3^{1–200}$/$Rnd1^{190–232}$) were cloned by PCR using pCMV5-Flag-Rnd3 and pRK5-Rnd1 (Nobes et al., 1998) as templates, and inserted into the *Eco*RI/*Hin*dIII sites of pCMV5-Flag. $Rnd3^{T55V}$, $Rnd3^{Y60A}$ and $Rnd3^{C241S}$ were created by site-directed mutagenesis (QuickChange kit, Stratagene). All constructs were verified by DNA sequencing (Eurofins MWG Operon). pCMV5-FLAG-Rnd3/2 ($Rnd3^{1–200}$/$Rnd2^{185–227}$) was a kind gift from Emilie Pacary (MRC National Institute for Medical Research, UK). pEGFP-RhoA, RhoB and RhoC were a gift from Ferran Valderrama (St George's University of London). pCMV2 or pmaxGFP (Lonza) were used as negative controls.

Cell culture and transfection

Pooled human umbilical vein endothelial cells (HUVECs; Lonza) were cultured in EGM-2 medium containing 2% foetal bovine serum (FBS). Prior to plating, cell culture dishes were coated with 10 µg/ml fibronectin (FN) for 1 hour at 37℃. HUVECs were used up to passage 4.

For DNA transfections, HUVECs were trypsinized, washed once in PBS, and resuspended at 10^6 cells/100 µl in HUVEC electroporation buffer (Amaxa, Cologne, Germany). Plasmid DNA (3 µg) was mixed with 100 µl of cell suspension, transferred to an electroporation cuvette, and transfected with an Amaxa Nucleofector (Amaxa) according to the manufacturer's instructions. After nucleofection, cells were immediately transferred to 1 ml of EGM-2 containing 2% FBS, and cultured at 37℃ until analysis.

For siRNA transfections, 10^5 HUVECs were plated per 6-well dish. After 24 hours, growth medium was replaced with Opti-MEM medium. Oligofectamine (Invitrogen) and siRNAs (100 nM) were incubated according to the manufacturer's instructions and added to the cells. After 4–6 hours the medium was replaced with HUVEC growth medium. Transfected cells were analysed after 48–72 hours.

HeLa cells were grown in DMEM supplemented with 10% donor calf serum (DCS) and penicillin/streptomycin (100 U/ml). HeLa cells were transfected with 3 µg of plasmid DNA using Lipofectamine 2000 (Invitrogen) in antibiotic-free Opti-MEM according to the manufacturer's instructions. The transfection medium was replaced with fresh DMEM containing 10% DCS 6 hours after transfection. Cells were analysed 24 hours after transfection.

mRNA isolation and RT-PCR

HUVECs were washed twice with PBS and then incubated for 5 minutes at room temperature with Trizol (Invitrogen) according to the manufacturer's instructions. Following incubation the lysate was transferred into a fresh tube. After addition of 0.1 ml chloroform and shaking, the mixture was centrifuged at 12,000 g for 15 minutes at 4℃. The upper phase was transferred to a new tube, 0.25 ml of isopropanol were added and incubated on ice for 10 minutes. After centrifugation at 12,000 g for 10 minutes at 4℃ the pellet was washed in 75% ethanol, air dried and resuspended in 20 µl H_2O. RNA was reverse-transcribed using SuperScript (Invitrogen), then the cDNA was amplified by PCR, using the following primers: Rnd1 fwd: 5′-CTATCCAGAGACCTATGTGCC-3′, rev: 5′-CGGACATTATCG-TAGTAGGGAG-3′ (122 bp); Rnd2 fwd: 5′-TCCTGATTCTGATGCTGTGCTC-3′, rev: 5′-ATTGGGGCAGAACTCTTGAGTC-3′ (106 bp); Rnd3 fwd: 5′-GAC-AGTGTCCTCAAAAAGTGGAAA-3′, rev: 5′-CTGGCGTCTGCCTGTGATT-3′ (139 bp). Products were separated on a 1% agarose gel.

Western blotting

Cells were lysed in 25 mM Tris-HCl pH 8, 130 mM NaCl and 1% NP-40 substitute, were applied to NuPAGE 4–12% Bis-Tris gels, and separated using NuPAGE MES running buffer (Invitrogen). Proteins were transferred to PVDF membranes (Immobilon-P, Millipore). Membranes were blocked in 5% skimmed milk powder or 3% BSA in TBST (50 mM Tris-HCl pH 7.7, 150 mM NaCl, 0.05% Tween), followed by incubation with primary antibodies in 2% skimmed milk powder/TBST or 3% BSA/TBST for 1 hour or overnight at 4℃. Membranes were then incubated with horseradish peroxidase-coupled secondary antibodies for 1 hour. Bound antibodies were detected by ECL (Amersham Bioscience UK). Signals were detected by exposure to X-Ray films.

Immunofluorescence and staining

For immunofluorescence labelling, HUVECs were grown on FN-coated glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at 4℃, and blocked with 3% BSA in PBS for 30 to 60 minutes. The cells were then incubated with primary antibodies in 3% BSA followed by incubation with AlexaFluor488- or AlexaFluor647-conjugated anti-rabbit or anti-mouse IgG secondary antibodies and/or AlexaFluor546-conjugated phalloidin to visualise F-actin. Coverslips were mounted onto slides using fluorescent mounting medium (DakoCytomation), and images acquired with a LSM 510 confocal laser scanning microscope (Zeiss, Welwyn Garden City, UK) using EC Plan-Neofluar 40×/1.30 and Plan-Apochromat 63×/1.40 oil-immersion objectives, and Zen software.

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Competing Interests

The authors have no competing interests to declare.

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